INTERRELATIONSHIPS AND CLINICAL SIGNIFICANCE OF EXPRESSION OF IMMUNOLOGICAL MARKERS IN INVASIVE BREAST CARCINOMA

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By

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DEDICATION

For my parents, Tom and Linda, who instilled in me a love of learning and have always been a constant support in my life.

ABSTRACT

The immunologic and prognostic significance of tumor cell HLA class II expression and immune cell infiltration in invasive breast carcinoma is unclear. The studies described in this thesis involved investigating several hypotheses regarding the anti-tumor immune response in invasive breast carcinoma in an attempt to provide clarification.

We postulated that the significance of tumor cell HLA-DR expression is dependent on HLA-DRB allotypes. In a pilot study, HLA-DR+ tumor cells differentially expressed HLA-DRβ allotypes, with DRβ1*04 preferentially expressed. An expanded study with clinicopathological and outcome data, confirmed these findings and demonstrated HLA-DRβ1*04 and HLA-DRβ1*13 expression by breast tumor cells, oppositely associate with survival. Evaluation of the intratumoral cytokine milieu suggested prognostic differences were attributable to variation in immune responsiveness, as HLA-DRβ1*04+ tumors had elevated T_H1-type cytokines, while DRβ1*13 expressing tumors had elevated immunoregulatory markers. In non-DRB1*04 tumors, lack of expression of one or more HLA-DRβ allotypes by HLA-DR+ tumors independently predicted decreased survival time, suggesting it may function in immune evasion.

Since *in vitro* studies demonstrated HLA-DR+ tumor cells function as antigen presenting cells, we evaluated the role of co-expression of the HLA class II co-chaperones, Ii and HLA-DM. DR+Ii+DM+ breast tumor cells independently predicted improved survival while DR+Ii+DM- tumors predicted decreased survival. Determination of intratumoral cytokine levels indicated improved prognosis of patients

with DR+Ii+DM+ tumors was attributable to enhanced T_H1-type immunity and elevated IFN-γ associated with improved survival.

As infiltrating inflammatory cells constitute the major source of immune-modulating cytokines, we correlated infiltrating cell subsets with cytokine mRNA levels. We observed the prognostic significance of tumor infiltrating cells is dependent on the balance of pro-inflammatory and immunoregulatory cytokines. A small exploratory study investigating whether genetic variation in cytokine genes partially regulate intratumoral cytokine responses demonstrated that allelic variation in cytokine gene regulatory sequences play a minor role controlling cytokine levels in breast tumors.

This expanded characterization of the *in situ* immune response in breast carcinoma enhances the understanding of anti-tumor immunity. This study suggests that future attempts to design and implement immunotherapeutic strategies in breast carcinoma patients should consider HLA-DRB allotypes, and involve co-incident enhancement of cell mediated immunity and suppression of immunoregulatory mechanisms.

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LIST OF ABBREVIATIONS AND SYMBOLS

AJCC	The American Joint Committee on Cancer
APC	Antigen presenting cell
B CA	Breast cancer
BCCL	Breast carcinoma cell lines
BCL	B cell lines
BiP	Immunoglobulin heavy chain binding protein
BLS	Bare lymphocyte syndrome
BRCA-1	Breast cancer 1 gene
BRCA-2	Breast cancer 2 gene
BRG1	Brahma-related gene 1
bp	base pairs
CARM-1	Co-activator-associated arginine methyltransferase 1
CAT	Chloramphenicol acetyltransferase
CBP	Cyclic-AMP-responsive-element-binding factor binding protein
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CIITA	Class II transactivator
CIITA-PI-IV	Class II transactivator promoter I-IV
CIIV	Multivesicular class II vesicles
CLIP	Class II associated invariant chain peptide
ConA	Concanavalin A
CREB	Cyclic-AMP-responsive-element-binding factor
CTL	Cytotoxic T lymphocyte
D	diffuse distribution
DC	Dendritic cell
DCIS	Ductal carcinoma *in situ*
DNA	Deoxyribonucleic acid
DMBA	7, 12-dimethylbenz(a)anthracene
dRFS	distant recurrence-free survival
DR51	HLA-DRβ5

DR52	HLA-DRβ3
DR53	HLA-DRβ4
DSS	disease-specific survival
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunoabsorbent assay
ER	Estrogen receptor
F	focal distribution
FF-PET	formalin-fixed paraffin embedded tissue
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAS	Interferon-gamma-activated site
GM-CSF	Granulocyte- macrophage colony-stimulating factor
HCMV	Human cytomegalovirus
HER	Human epidermal growth factor receptor
Her-2/neu	Human epidermal growth factor receptor-2
HLA	Human leukocyte antigen
HR	hazards ratio
HRP	horseradish peroxidase
I	Intratumoral T cells
IFN-γ	Interferon gamma
IDC	Invasive ductal carcinoma
IDC NST	Invasive ductal carcinoma, no special type
IHC	Immunohistochemistry
IHW	International Histocompatibility Workshop
Ii	Invariant chain
ILC	Invasive lobular carcinoma
IL	Interleukin
IRF	Interferon regulatory factor
IRFE	Interferon regulatory factor element
JAK	Janus kinase
JNK	c-Jun N-terminal kinase

KLH	Keyhole limpet hemocyanin
LCIS	Lobular carcinoma *in situ*
LCM	Laser capture microdissection
LMP	Low molecular weight protein
LN	Lymph Node
LOH	Loss of heterozygosity
LPS	lipopolysaccharide
LR	log rank statistic
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
Mb	Megabase pairs
MBTB	Manitoba breast tumor bank
MHC	Major Histocompatibility Complex
MICA	MHC class I-related chain A
MICB	MHC class I-related chain B
MIIC	MHC class II loading compartments
MMTV	Murine mammary tumor virus
mRNA	Messenger ribonucleic acid
NF-Y	Nuclear factor binding to the Y-box
NK	Natural killer cell
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCAF	p300/CBP-associated factor
PCR-SSP	Polymerase chain reaction with sequence specific primers
PHA	phytohemagglutinin
PI3K	phosphoinositide 3-kinase
PMA	phorbol-myristate acetate
PR	Progesterone receptor
P-TEFb	Positive transcription elongation factor b
PTEN	Phosphatase and tensin homolog
RFS	recurrence-free survival

RFX	Regulatory factor X
RFXANK	Regulatory factor X associated ankyrin-containing protein
RFX5	Regulatory factor X 5
RFXAP	Regulatory factor X associated protein
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Stromal T cells
SD	Standard deviation
SE	Standard error
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TA	Tumor antigen
TAA	Tumor-associated antigen
TAM	Tumor-associated macrophages
TAP	Transporter associated with antigen processing
TCL	T cell line
TCR	T cell receptor
TFIIB	Transcription factor IIB
TFIID	Transcription factor IID
TGF-β	Transforming growth factor beta
TIL	Tumor infiltrating lymphocytes
TNF-α	Tumor necrosis factor alpha
TNM Stage	Tumor Stage determined by tumor size (T), axillary lymph node status (N) and presence or absence of distant metastasis (M)
TP53	Tumor protein p53
T_{reg}	regulatory T cell
USF	Upstream transcription factor
VEGF	Vascular endothelial growth factor
ZAP-70	Zeta-chain-associated protein kinase
$\bar{\times}_{\%}$	Mean percentage of positive tumor cells ± standard deviation

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION AND OVERVIEW

1.1 Preface

The following literature review is divided into subsections that will provide the reader with a comprehensive summary of established prognostic indicators of breast carcinoma, Human Leukocyte Antigens (HLA) and anti-tumor immune responsiveness. The first part will describe the prognostic and predictive nature of clinicopathological parameters in breast carcinoma. HLA antigens will then be reviewed with a focus on the genetic complexity of HLA class II genes, control of HLA class II gene expression, the function of classical and non-classical HLA class II molecules and the related co-chaperone invariant chain (Ii) in antigen presentation and the influence of HLA class II polymorphisms on immune responsiveness. The remaining sections will highlight literature that has attempted to elucidate the biological and prognostic significance of HLA class II antigens in breast carcinoma and provide an overview of immune responsiveness in breast carcinoma.

1.2 Breast Carcinoma

The mature adult breast is comprised of 15-25 lobes each associated with lactiferous ducts, which originate from the nipple. Each lobe of the breast consists of a system of branching ducts, embedded in hormone responsive stroma. At the onset of puberty, elevated levels of estrogen, progesterone, insulin and growth hormone are responsible for ductal outgrowth and lobuloalveolar differentiation. Lobules develop at the ends of the terminal ducts, resulting in the formation of terminal ductal-lobular units [1]. The majority of hyperplastic and neoplastic breast lesions develop following

epithelial transformation in the lining of the terminal ductolobular unit [2]. *In situ* carcinomas are non-invasive and contained within the basement membrane of either the duct (ductal carcinoma *in situ*, DCIS) or lobule (lobular carcinoma *in situ*, LCIS). Once the malignant cells have invaded the basement membrane tumors are classified as invasive [3].

1.3 Evaluation of Prognosis and Outcome in Invasive Breast Carcinoma

Several clinicopathological factors have been defined for breast carcinoma, which function as predictive and/or prognostic indicators. Predictive factors determine response to a particular therapy and include the hormone receptors, estrogen receptor (ER) and progesterone receptor (PR), and human epidermal growth factor receptor-2 (Her-2/neu). These factors are also prognostic indicators as they are used to estimate the risk of disease recurrence or death, independent of adjuvant treatment. Other factors which estimate patient outcome are the histological type and grade of the breast tumor, tumor diameter, axillary lymph node involvement and the presence of metastasis to distant sites within the body.

1.3.1 Histological Tumor Type

Invasive breast carcinomas largely arise from transformation of epithelial cells lining the ducts and are termed invasive ductal carcinomas (IDC). Special subtypes of IDC are classified based on the architectural growth pattern of the malignant cells. For example, the malignant cells of tubular breast carcinomas display a tubular growth pattern, cribiform carcinomas are marked by islands of invasive tumor cells forming arches outlining well-defined spaces, mucinous tumors contain malignant cells

surrounded by an extracellular matrix of mucin, and medullary carcinomas display a sheet-like growth pattern. IDC that do not satisfy the criteria for classification in other categories are defined as IDC no special type (IDC NST) and comprise 50-70% of all invasive breast cancers [3]. The second most common form of invasive breast carcinoma is invasive lobular carcinoma (ILC), which originates in the breast lobule [3]. Patients with ILC have improved survival as compared to those with IDC NST [3, 4]. Likewise, patients with the aforementioned subtypes of IDC have prolonged disease-free and overall survival as compared to IDC NST [3].

1.3.2 Histological Grade of Tumor Differentiation

Most classification schemes for the grade of tumor differentiation are based on the scheme of Bloom and Richardson, which assigns qualitative scores of 1-3 for each of tubule formation, nuclear pleomorphism and mitotic rate. The three scores are combined to assign a tumor grade from 3-9, and tumors are further divided into either low (Grade I, scores 3-5), intermediate (Grade II, scores 6-7) or high (Grade III, scores 8-9) tumor grade. In this large scale study, patients with Grade I tumors show improved 5, 10- and 15-year survival over patients with Grade II and III tumors [5]. The commonly used Nottingham modification of this grading system uses a semi quantitative assessment of each of these three factors (Table 1.1) to create a more objective scoring system for histological grade. Tumors are classified as well-differentiated (Grade I, scores 3-5), moderately differentiated (Grade II, scores 6-7) or poorly differentiated (Grade III, scores 8-9). Similar to the results of Bloom and Richardson (1957), using this system, patients with grade I tumors show significantly improved disease-free and overall survival than those with grades II or III [6].

Table 1.1: Nottingham/Tenovus Primary Breast Cancer Study semi quantitative method for assessment of histological grade in breast carcinoma*

Feature	Classification	Mitotic Count†	Score
Tubule formation	Majority of tumor (>75%)		1
	Moderate amount (10-75%)		2
	Little or none (<10%)		3
Nuclear pleomorphism	Small, regular uniform cells		1
	Moderate increase in size and variability		2
	Marked variation		3
Mitotic counts (dependent on microscope field – representative examples shown)	Leitz Ortholux (250X; field area 0.274mm^2)	0-9	1
		10-19	2
		>20	3
	Nikon Labophot (400X; field area 0.152mm^2)	0-5	1
		6-10	2
		>11	3
	Leitz Diaplan (400X; field area 0.312mm^2)	0-11	1
		12-22	2
		>23	3

*Table modified from Elston and Ellis (1991) [6].

†# of mitotic cells per 10 microscope fields at the tumor periphery.

1.3.3 Tumor Size, Lymph Node Status and TNM Tumor Stage

Two of the most important prognostic parameters for breast carcinoma are regional lymph node (LN) status and the diameter of the tumor. In a large scale study, stratification of tumors by tumor size (< 2cm, between 2 and 5cm and larger than 5 cm) or lymph node involvement (negative, 1-3 involved nodes and 4 or more involved nodes) show 5-year survival rates are highest when the tumor is <2 cm and there is no LN involvement. Furthermore, 5-year survival decreased with increasing tumor size or increasing LN involvement and these two parameters were shown to act as independent but additive prognostic indicators [7].

The American Joint Committee on Cancer (AJCC) has established an in depth pathological staging system which takes into account tumor size (T), the number of involved axillary lymph nodes (N), and presence or absence of distant metastasis (M) (Table 1.2) [8]. Using the revised AJCC classification system, a clear difference in survival is observed based on the number of involved axillary lymph nodes. The 5-year disease-free survival rates are 69.5% for the N1 group, 46.8% for the N2 group, and 25.7% for the N3 group. Likewise, disease-free survival rates are progressively worse with increasing TNM stage [9].

Assessment of tumor size may be complicated by the occurrence of multiple tumor foci in the same breast, in such cases it is recommended that tumor size be recorded as the largest foci diameter [8]. However, tumor multifocality is prognostic as it predicts local cutaneous recurrences in patients with stage I or II tumors, treated with breast-conserving therapy [10].

Table 1.2: AJCC Stage Groupings for breast carcinoma

TNM Stage	Classification Category		
	Tumor Size*	LN status†	Metastasis‡
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

* Tis = Carcinoma *in situ*; T0 = no evidence of primary tumor; T1 = ≤2cm; T2 = 2.1-5cm; T3 = >5cm; T4 = any size with direct extension to the chest wall or skin or inflammatory cancer.

† N0 = No regional lymph node metastasis; N1 = Metastasis in 1-3 axillary lymph nodes; N2 = Metastasis in 4-9 axillary lymph nodes; N3 = Metastasis in ≥10 axillary lymph nodes

‡ M0 = No distant metastasis; M1 = Distant metastasis

1.3.4 Hormones and Hormone Receptors

A role for hormones in the development of breast cancer is suggested by the fact that breast cancer incidence begins to increase late in the third decade of life, whereas the incidence rates for other common cancers (e.g. colorectal) start to increase in the late 40s. This difference has been attributed to the responsiveness of breast epithelium to ovarian hormones, which are elevated from puberty to menopause [11]. It has long been recognized that early oophorectomy leads to decreased breast cancer risk [12] and serum estrogen levels are higher in breast carcinoma patients than controls [13]. Further indirect evidence for a role of prolonged hormone exposure in elevated breast cancer risk is provided by the fact that breast cancer incidence is increased in women who experience early menarche ($\leq$ 12 years) [14], late menopause (>55 years as compared to <45 years) [12], later age of first pregnancy (>35 years as compared to <20 years) [15] and nulliparity [14]. Experimental evidence is provided by rodent studies where long term exposure to high doses of either 17β-estradiol or synthetic progesterone leads to the development of malignant mammary tumors [16, 17].

The hormone receptors, ER and PR, are members of the nuclear receptor subfamily of transcription factors [18]. Following hormone binding and dimerization they translocate to the nucleus where they activate or repress target gene transcription [18]. ER and PR are important for normal breast development as evidenced by lack of epithelial ductal outgrowth in ER knockout mice [19] and the lack of alveolar development in PR knockout mice [20]. The importance of ER and PR in breast tumorigenesis is illustrated by the decreased incidence of mammary tumors in PR and ER knockout mice

administered tumorigenic doses of the carcinogen 7, 12-dimethylbenz(a)anthracene (DMBA) [21, 22].

Presence of ER and PR predicts response to hormone treatment as tumors that express both hormone receptors show marked regression following endocrine therapy, with tamoxifen accounting for the majority of treatments [23]. Likewise, both function as prognostic indicators as illustrated by a recent population-based cohort study of over 155,000 breast carcinoma patients with up to 11 years of follow-up. Hormone receptor status functioned as an independent prognostic factor after controlling for other prognostic variables and patients with ER^+PR^+ tumors had the best survival rates, while ER^-PR^- tumors associated with the lowest survival rates [24].

1.3.5 Her-2/neu

The HER (human epidermal growth factor receptor) family of receptors includes epidermal growth factor receptor (EGFR or HER-1 or *erb*-B1), HER-2 (Her-2/neu or *erb*-B2), HER-3 (or *erb*-B3) and HER-4 (or *erb*-B4). HER receptors exist as inactive monomers that form hetero- or homodimers upon ligand binding resulting in cross-phosphorylation of their cytoplasmic tyrosine kinase domains, signal transduction to the nucleus, nuclear gene activation and cell growth [25].

Her-2/neu is a proto-oncogene located on Chromosome 17q21 which encodes a 185 kD transmembrane glycoprotein with extensive homology to EGFR [26]. Early studies using Southern blotting reported Her-2/neu is over expressed in up to 30% of breast tumors [27]. Her-2/neu expression has been evaluated using numerous techniques including Southern blotting, immunohistochemistry with specific monoclonal antibodies, enzyme-linked immunoabsorbent assay (ELISA), reverse transcriptase polymerase chain

reaction (RT-PCR) and *in situ* hybridization, and the proportion of breast tumors with over amplification of the Her-2/neu gene or over expression of the Her-2/neu protein has been reported between 10% to 34% [28]. The most common cause of over expression of Her-2/neu protein is gene amplification resulting in 2 to 100-fold amplification as assessed by Southern blotting and densitometry, normalized to collagen genes [29]. Her-2/neu over-expression is associated with histological unfavorable micropapillary and apocrine breast carcinomas [30], apoptosis inhibition and increased cell proliferation, via phosphoinositide 3-kinase (PI3K) activation and suppression of p53 [31], increased mitogen-activated protein kinase (MAPK) activation [32], increased anchorage independent growth and metastatic potential [33], and increased vascular endothelial growth factor (VEGF) expression and angiogenesis [34]. The prognostic power of Her-2/neu expression is evident as membrane over-expression of the Her-2/neu protein independently predicts poor relapse-free and overall survival, after controlling for the effects of LN metastasis, tumor size, diagnosis age and hormone receptor status [27].

Expression of a rat *neu* proto-oncogene, with an activating mutation in the transmembrane region, under control of the murine mammary tumor virus (MMTV) promoter leads to spontaneous development of mammary tumors in mice [35]. A MMTV/*neu* transgene without the activating mutation also resulted in Her-2/neu over expression, increased tyrosine kinase activity and mammary tumor development. The kinetics of tumor development were slower than that seen with the activated transgene but a high percentage of mice also developed metastatic lung tumors [36]. A later study revealed spontaneous somatic mutations in wild type *neu* in this model, leading to its over-expression [37].

Her-2/neu was initially considered a predictive factor as its over-expression resulted in poor response to tamoxifen treatment in a long-term (median follow-up 12 years) randomized controlled clinical trial [38]. This was likely an indirect association, attributable to the negative correlation observed between Her-2/neu and ER expression. Certainly, Her-2/neu over-expression failed to predict poor response to tamoxifen therapy in ER positive breast cancer patients [39]. More recently, an anti-Her-2/neu humanized antibody, trastuzumab (Herceptin®), has been evaluated for beneficial efficacy in breast carcinoma. Slamon et al. (2001) demonstrated improved response rate, prolonged time to progression and overall survival in metastatic breast carcinoma patients treated with chemotherapy and trastuzumab as compared to those administered chemotherapy alone [40]. The results of large multi-center trials revealed significantly decreased risk of recurrence in Her-2/neu-positive breast cancer patients treated with trastuzumab combined with or following chemotherapy treatment [41, 42]. However, it is important to note that the results of these studies represent that of the interim efficacy analyses and further follow-up of patients may yield varying risks.

1.3.6 Age at diagnosis

Young women (<35/<40 years) diagnosed with breast cancer more frequently present with tumors that lack expression of ER and PR and are poorly differentiated [43, 44]. The prognostic value of age at diagnosis remains unclear. Some studies reported no difference in survival between early onset and late onset breast carcinoma [45, 46], while others found improved survival in patients diagnosed at an early age [47]. However, after controlling for other prognostic factors and treatment, numerous studies identified early age at diagnosis as an independent risk factor for poor survival [4, 48-50]. Increased

levels of circulating hormones in young women may contribute to carcinogenesis by increasing cell proliferation, allowing for accumulation of genetic errors.

The more aggressive tumor phenotype and decreased survival of young women with breast cancer may in part be due to increased incidence of germline mutations in these patients. Approximately 40% of women diagnosed before age 40 years have a family history of breast or ovarian cancer [51] and 10% of those carry germline mutations in the carcinoma predisposing genes, breast cancer (BRCA)-1 or BRCA-2 [51, 52]. In addition, germline BRCA-1 mutations are associated with high tumor grade, ER-negativity and decreased survival of breast cancer patients [53]. Germline mutations in the tumor suppressor gene E-cadherin located on chromosome 16 are frequent in lobular breast carcinomas [54]. Increased risk for early-onset breast carcinoma is also associated with Li-Fraumeni syndrome, which is caused by a dominant mutation in the tumor suppressor gene TP53 (tumor protein p53) located on chromosome 17, and Cowden's syndrome, which is caused by a mutation in the tumor suppressor gene PTEN (phosphatase and tensin homolog) located on chromosome 10 [55].

A role for HLA genes, which are key regulators of immunity, in early onset breast carcinoma is suggested by the case-control study of Chaudhuri et al. (2000) who reported that HLA-DRB1*11 and HLA-DQB1*0303 were decreased in early onset breast carcinoma patients ($\leq$40 years), as compared to age-matched healthy controls. Although these associations may be indirect and attributable to linkage of these HLA class II alleles with unidentified tumor suppressor genes, other HLA-DRB1 alleles known to be carried with HLA-DQB1*0303 did not associate with disease protection. This suggests the association may reflect an influence of these alleles on anti-tumor immune surveillance,

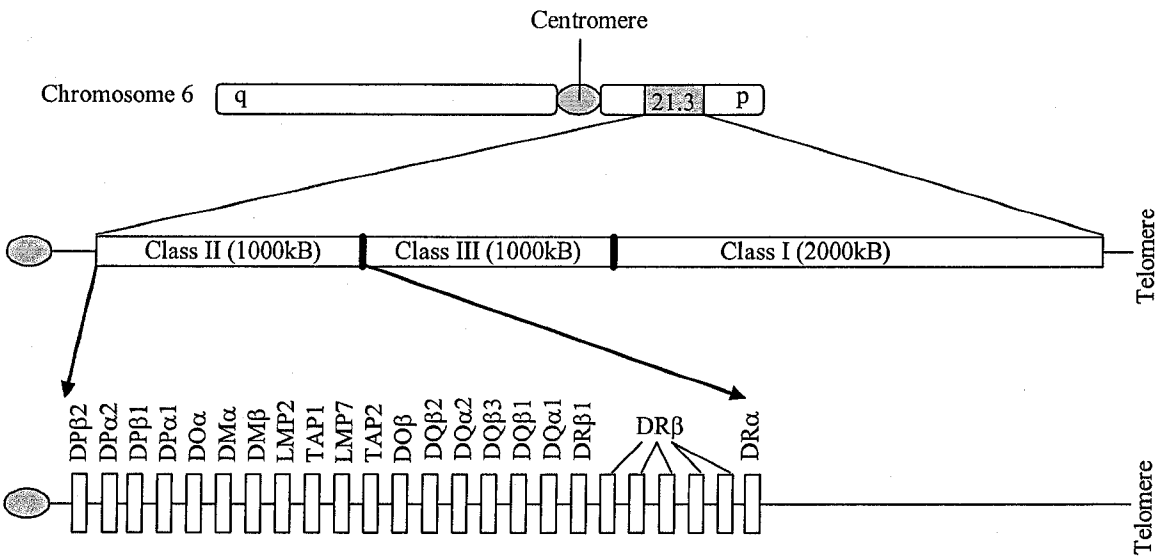
as HLA class II alleles encode glycoproteins that present antigens to CD4+ T cells, which are the central players in immune responsiveness.

1.4 The Major Histocompatibility Complex

The Major Histocompatibility Complex (MHC) is a collection of genes contained within a long segment of DNA and referred to as the H-2 complex in mice and the HLA complex in humans. Both complexes are organized into groups of genes that code for three classes of molecules. Class I and Class II genes encode cell surface glycoproteins that bind and present peptides to CD8+ and CD4+ T cells, respectively. Class III genes encode molecules crucial for immunity but share no structural similarity to the MHC class I or II molecules. Due to their role in antigen presentation class I and class II molecules are termed classical MHC molecules. MHC class I molecules are encoded by the K and D loci in mice and A, B and C loci in humans, while MHC class II molecules are encoded by the I-A and I-E regions in mice and the DR, DP and DQ regions in humans [56].

1.4.1 Human Leukocyte Antigens

The HLA complex is located on Chromosome 6p21.3 and is the human form of the MHC, which contains many genes, involved in innate and adaptive immunity. Recently, an extended human MHC map has been published which spans 7.6 mega base (Mb) pairs and includes 252 expressed genes and 139 pseudogenes [57]. The first gene map of the HLA complex, termed the classical human MHC was 3.6 Mb and contained 224 loci [58]. As mentioned above, the classical human MHC is organized into three classes (Figure 1.1). The HLA designation is given only to those genes in the class I and



Adapted from Goldsby, Kindt and Osborne, 2000

Figure 1.1: The HLA complex is located on the short arm of Chromosome 6 (6p21.3) and is organized into three regions based on Class. The HLA class II region spans 1000 kB and encodes for the classical HLA class II (DP, DQ, DR) molecules and the non-classical HLA-DM and HLA-DO molecules, as well as the TAP and LMP molecules involved in the HLA class I pathway. Figure adapted from Goldsby, Kindt and Osbourne (2000) [56].

class II regions that encode class I and II glycoproteins (often referred to as allotypes) involved in antigen presentation and their related pseudogenes [59].

The classical HLA Class I genes (HLA-A, -B, -C) encode glycoproteins expressed by nearly all nucleated cells that form heterodimers with the monomorphic β_2-microgloblin protein and present endogenously derived peptides of 8-10 amino acids in length to $CD8^+$ T cells for recognition [59]. The class I region also encodes the non-classical HLA genes (HLA-E, -F, -G) [58], which function as natural killer (NK) cell inhibitory ligands [60, 61] and the MHC class I-related chain A (MICA) and MICB [58], which act as NK activating ligands [62].

The HLA class III region encodes a series of genes that are not involved in direct antigen presentation but still play a role in regulating immune responses, including tumor necrosis factor (TNF)-α, lymphotoxin and the complement factors C2 and C4 [58].

Classical HLA class II genes (HLA-DPA, -DPB, -DQA, DQB, -DRA and -DRB) encode class II α chain and β chain glycoproteins that form heterodimers and present exogenously-derived peptides, of 12-24 amino acid residues in length, to $CD4^+$ T cells for recognition [59]. HLA class II antigens are constitutively expressed by thymic epithelial cells and the professional antigen presenting cells (APC), B cells, dendritic cells (DC), and macrophages but can be up-regulated in so called non-professional antigen presenting cells (e.g., keratinocytes, epithelial cells, endothelial cells and fibroblasts), most notably in response to interferon gamma (IFN-γ) [63-65]. The class II region also encodes the non-classical HLA-DM and HLA-DO antigens [58], which act as co-chaperones facilitating peptide loading of HLA class II molecules [66, 67] and the

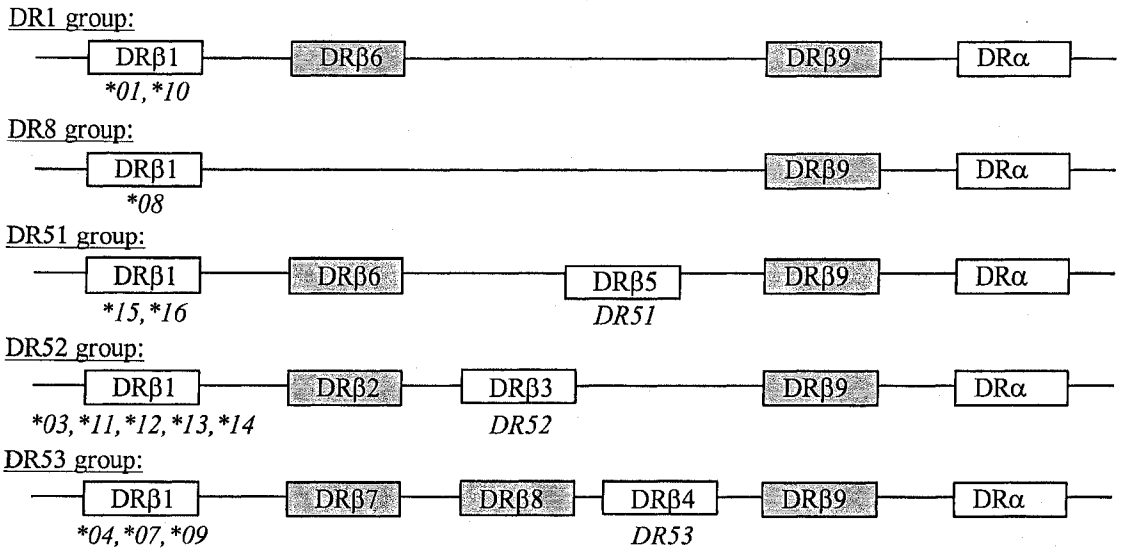
transporter associated with antigen processing (TAP) and low molecular weight protein (LMP) molecules [58], involved in the cytosolic HLA class I processing pathway [68].

Classical HLA class II molecules are highly polymorphic. HLA-DRA is considered monomorphic, with minimal variation between the DRA alleles and the polymorphism of HLA-DR antigens is attributable to the HLA-DRβ chain. In contrast, both α and β chains contribute to the polymorphism of HLA-DP and HLA-DQ antigens and the non-classical HLA-DM and HLA-DO isoforms exhibit little polymorphism [59].

1.4.2 HLA class II antigens

The IMGT/HLA database has identified 3 DRA, 430 DRB (354 DRB1, 1 DRB2, 39 DRB3, 12 DRB4, 17 DRB5, 3 DRB6, 2 DRB7, 1 DRB8, and 1 DRB9), 25 DQA1, 56 DQB1, 20 DPA1 and 106 DPB1 alleles [69]. The genes that encode the HLA α and β chains are designated as A and B, respectively. HLA-DQ is encoded by HLA-DQA1 and HLA-DQB1 and there are also HLA-DQA2, HLA-DQB2 and HLA-DQB3 pseudogenes. HLA-DP is encoded by HLA-DPA1 and HLA-DPB1 and there are also HLA-DPA2 and HLA-DPB2 pseudogenes. HLA-DM is encoded by HLA-DMA and HLA-DMB, HLA-DO by HLA-DOA and HLA-DOB [59].

The HLA class II region that encodes HLA-DR molecules is far more complex due to the extraordinarily high number of allelic variants at the HLA-DRB1 locus, most of which are in linkage with additional structural HLA-DRB genes [70]. HLA-DRB1*15 and 16 alleles are expressed in association with HLA-DRB5 alleles (DR51); HLA-DRB1*03, 11, 12, 13 and 14 with HLA-DRB3 alleles (DR52); HLA-DRB1*04, 07 and 09 with HLA-DRB4 (DR53); while HLA-DRB1*01, 10 and 08 are not in association with any other expressed HLA-DRB genes (Figure 1.2). Thus, resultant from the tight



Adapted from Campbell and Trowsdale, 1993

Figure 1.2: HLA-DR alleles are grouped into 5 haplotype groups based on shared DRβ genes. Pseudogenes are indicated by grey boxes. Figure adapted from Campbell and Trowsdale (1993) [70].

linkage of DRB genes and co-dominant expression of HLA loci, an individual can express up to four different HLA-DR allotypes. Furthermore, the tight linkage between HLA-DR and HLA-DQ genes results in a limited number of haplotypes (Table 1.3).

1.4.3 HLA Class II Antigen Processing and Presentation Pathway

HLA class II α and β chains are synthesized in the rough endoplasmic reticulum and exist briefly in high molecular weight aggregates containing immunoglobulin heavy chain binding protein (BiP) [71]. These aggregates then assemble into Ii containing oligomers associated with calnexin, a chaperone protein [72]. The association between Ii and class II molecules in the endoplasmic reticulum is important in stabilizing correctly folded class II molecules, through the formation of a nine-subunit $(\alpha\beta)_3Ii_3$ complex which is transported from the endoplasmic reticulum [73]. Binding of Ii also prevents premature binding of antigenic peptides while in the endoplasmic reticulum and during transport to the endosomes [74].

The release of calnexin corresponds with the formation of complete nonameric $(\alpha\beta)_3Ii_3$ complexes [72, 75]. These nonameric complexes are transported through the Golgi apparatus and trans-Golgi network to endocytic compartments, including early endosomes, late endosomes and lysosomes. The greatest abundance of intracellular MHC class II is in late endosome/lysosome-like compartments, which are termed MHC class II loading compartments (MIIC) [76]. Within MIIC, Ii is degraded and a class II-associated Ii peptide (CLIP) remains bound to the peptide binding groove [77]. Peptide loading often occurs within MIICs and can also occur in multivesicular class II vesicles (CIIV), which more closely resemble late endosomes [78].

Table 1.3: HLA-DRB1-DQB1 Haplotype Groups in European Americans[†]

HLA-DRB Haplotype Group	Linked DRβ	DRβ1	HLA-DR Serological Specificities[‡]	Associated DQβ1[‡]	HLA-DQ Serological Specificities[‡]
DR1/10	-	0101	DR1	05	DQ5
		0102	DR1	05	DQ5
		0103	DR103	*05/0301*	DQ5/*DQ7*
		1001	DR10	05	DQ5
DR8	-	0801	DR8	*04/0301//0302*	DQ4/*DQ7/DQ8*
		0802	DR8	04	DQ4
		0803	DR8	0301	DQ7
		0804	DR8	04	DQ4
DR51	DRβ5	1501	DR15 (2)	*06/05*	DQ6/*DQ5*
		1502	DR15 (2)	06	DQ6
		1503	DR15 (2)	06	DQ6
		1601	DR16 (2)	05	DQ5
		1602	DR16 (2)	*05/0301*	DQ5/*DQ7*
DR52	DRβ3	0301	DR17 (3)	*02/0301/06*	DQ2/DQ7/DQ6
		1101	DR11	*0301/0302/06*	DQ7/*DQ8/DQ6*
		1102	DR11	0301	DQ7
		1103	DR11	0301	DQ7
		1104	DR11	*0301/05/06*	DQ7/DQ5/DQ6
		1201	DR12	0301	DQ7
		1301	DR13 (6)	*06/05*	DQ6/*DQ5*
		1302	DR13 (6)	06	DQ6
		1303	DR13 (6)	0301	DQ7
		1305	DR13 (6)	0301	DQ7
		1401	DR14 (6)	*05/06*	DQ5/*DQ6*
		1404	DR14 (6)	05	DQ5
DR53	DRβ4	0401	DR4	0301/0302*/04*	DQ7/DQ8/*DQ4*
		0402	DR4	0302	DQ8
		0403	DR4	*0302/0304/0305*	DQ8/*DQ7/DQ8*
		0404	DR4	*0302/02/04*	DQ8/*DQ2/DQ4*
		0405	DR4	*0302/02*	DQ8/*DQ2*
		0406	DR4	04	DQ4
		0407	DR4	*0301/0302*	DQ7/*DQ8*
		0408	DR4	*0301/0304*	DQ7/*DQ7*
		0701	DR7	*02/0303/0301*	DQ2/DQ9/*DQ7*
		0901	DR9	*0303/02*	DQ9/*DQ2*

[†]Table created from information in Klitz et al (2003) [79], European American defined as self-described as Caucasian, White or European origin. Infrequent haplotypes indicated by italics. A total of 1899 individuals with 3798 haplotypes were molecularly typed by PCR-SSP for HLA-DR and HLA-DQ alleles.
[‡]Serological specificities obtained from Schreuder et al (2005) [80]. Numbers in parenthesis represent older serological designations.

In normal APC, CLIP is exchanged for an exogenously derived antigenic peptide that is loaded into the peptide-binding groove, via the action of the co-chaperone HLA-DM. Cells lacking HLA-DM molecules do not undergo this exchange and CLIP remains bound within the groove [81]. HLA-DM enhances the rate of removal of CLIP indicating that it is a necessary molecule for active removal [66, 82]. However, HLA-DM negative cells can self-release CLIP enabling them to bind exogenous antigens [83]. HLA-DM may act as a selecting agent for epitope binding, removing kinetically unstable peptides, and allowing for binding of only high-stability peptides [84].

The HLA class II molecule HLA-DO forms stable complexes with HLA-DM in B cells, with HLA-DM being required for transport of HLA-DO from the endoplasmic reticulum to lysosomes, where both molecules are found [85]. HLA-DO preferentially promotes loading of HLA class II molecules that depend on HLA-DM, thereby acting as a co-chaperone of HLA-DM, controlling the binding of antigenic peptides that are to be presented at the cell surface. In this role, HLA-DO influences peptide editing in a positive way for some peptides and negatively for others. In particular, HLA-DO was found to enhance the efficiency of peptide loading of class II molecules such as HLA-DRβ1*04 and DRB1*01 over HLA-DM alone, but had no effect on HLA-DRB1*03 molecules [67], while over expression of HLA-DO leads to the accumulation of HLA-DR-CLIP complexes at the cell surface of a HLA-$DR^{+}DM^{+}$ human T cell line [86].

HLA class II molecules classically present peptides derived from exogenous antigens, however, peptides generated from cytosolic antigens have also been eluted from the peptide-binding groove [87]. The source of HLA class II peptides is also influenced by cell type as epithelial cells transfected with HLA-DRβ1*04, Ii and HLA-DM have a

more diverse epitope repertoire and display more cytoplasmic-derived peptides than B lymphoblastoid cell lines [88]. The identification of tumor cell activation of tumor antigen specific HLA class II restricted $CD4^+$ T cells [89] highlights the importance of endogenous antigen presentation by HLA class II molecules. Immune escape mechanisms employed by viruses that block this phenomenon further emphasize its importance. For example, the human cytomegalovirus (HCMV) carries two glycoproteins, which inhibit the HLA class II pathway preventing *in vitro* activation of $CD4^+$ T cells. The glycoprotein US2 binds to and causes the degradation of DRα and DMα chains and the glycoprotein US3 binds newly-synthesized HLA class II αβ dimers inhibiting their interaction with Ii and causing decreased accumulation of HLA class II in peptide-loading compartments [90]. As these glycoproteins are only expressed in virally infected cells they likely evolved to block the presentation of endogenous viral antigens by HLA class II molecules to anti-HCMV $CD4^+$ T cells.

1.4.4 Regulation of HLA class II Expression

HLA class II gene expression is tightly regulated, underlying the restricted expression of HLA class II antigens to professional APC and thymic epithelial cells. The induction of HLA class II expression in other cell types is also tightly regulated, occurring in response to exogenous signals, most notably IFN-γ produced during infection and/or inflammation.

1.4.4.1 HLA class II promoter elements and trans-acting transcription factors

The promoters of all HLA class II, Ii, HLA-DM and HLA-DO genes contain a conserved SXY motif, which binds DNA transcription factors to promote gene

expression. Substantial information regarding MHC class II regulation was obtained from investigation of the mutations responsible for the MHC class II immunodeficiency, bare lymphocyte syndrome (BLS). Cell-fusion experiments performed with MHC class II-negative cell lines derived from BLS patients revealed BLS patients could be grouped into one of 4 complementation groups (A-D), based on the ability to restore MHC class II expression in the hybrid cells [91]. Restoration of HLA class II expression in B cell lines from BLS patients by transfection with cDNA libraries and DNA sequencing confirmed the identification of the four mutated genes. BLS complementation groups A-D, carry mutations in the MHC class II transactivator (CIITA) [92], regulatory factor X (RFX)-associated ankyrin-containing protein (RFXANK) [93], RFX5 [94] and RFX associated protein (RFXAP) [95], respectively.

The SXY region is bound by *trans*-acting factors, which mediate HLA class II transcription (Figure 1.3). In *vitro* assays have shown the S-box can bind RFX, however, the function of the S-box in recruiting CIITA to the enhanceosome is mediated by an unidentified DNA binding transcription factor [96]. The X-box is bound by RFX5 [94], RFXANK [93] and RFXAP [95] which form the trimeric RFX complex. The X2-box is bound by cyclic-AMP-responsive-element-binding factor (CREB) [97]. The Y-box is bound by the nuclear factor binding to the Y-box (NF-Y) protein [98]. This multiple protein HLA class II regulatory module formed by binding of trans-acting proteins to the SXY motif is termed the enhanceosome [99].

1.4.4.2 The MHC Class II Transactivator (CIITA)

CIITA is non-DNA-binding but acts as a scaffold, binding RFX, CREB and NF-Y proteins within the enhanceosome [100] (Figure 1.3). CIITA then coordinates gene

transcription via the recruitment of proteins involved in histone acetylation (CREB-binding protein (CBP), p300 and p300/CBP-associated factor (PCAF)), methylation (co-activator-associated arginine methyltransferase 1 (CARM1), chromatin remodeling (brahma-related gene 1 (BRG1), transcription initiation (transcription factor IID (TFIID) and TFIIB) and transcription elongation (positive transcription elongation factor b (P-TEFb)) [101]. CIITA transcription is tightly controlled by three independent promoter units (CIITA-PI, -PIII and –PIV), each transcribing a different first exon and resulting in three CIITA isoforms with differing amino-termini. A fourth promoter (CIITA-PII) is also found in the human CIITA gene, but its function is not yet known [102].

The functions of the three CIITA promoters were determined by assessing the phenotype of mice with targeted deletions of the CIITA gene. A targeted deletion that excised CIITA-PIV and its associated first exon but left CIITA-PI and CIITA-PIII intact [103] revealed that CIITA-PIV was essential for IFN-γ-induced MHC class II expression in non-hematopoietic cells (epithelial cells, endothelial cells, astrocytes and fibroblasts). However, constitutive MHC class II expression was not altered in B cells, DC or tissue macrophages and IFN-γ induction of MHC class II expression on peritoneal macrophages and migroglial cells was unaltered, indicating CIITA-PIV is not required for this process. These mice also have impaired positive selection of $CD4^+$ T cells, owing to the lack of expression of MHC class II on cortical thymic epithelial cells. However, the role of CIITA-PIV in MHC class II expression by thymic epithelial cells is likely independent of IFN-γ, as $CD4^+$ T cell positive selection is not abolished in mice lacking IFN-γ or the IFN-γ receptor [104, 105]. A second strain of mice contains a deletion that excises CIITA-PIII and CIITA-PIV and their associated first exons but leaves CIITA-PI intact

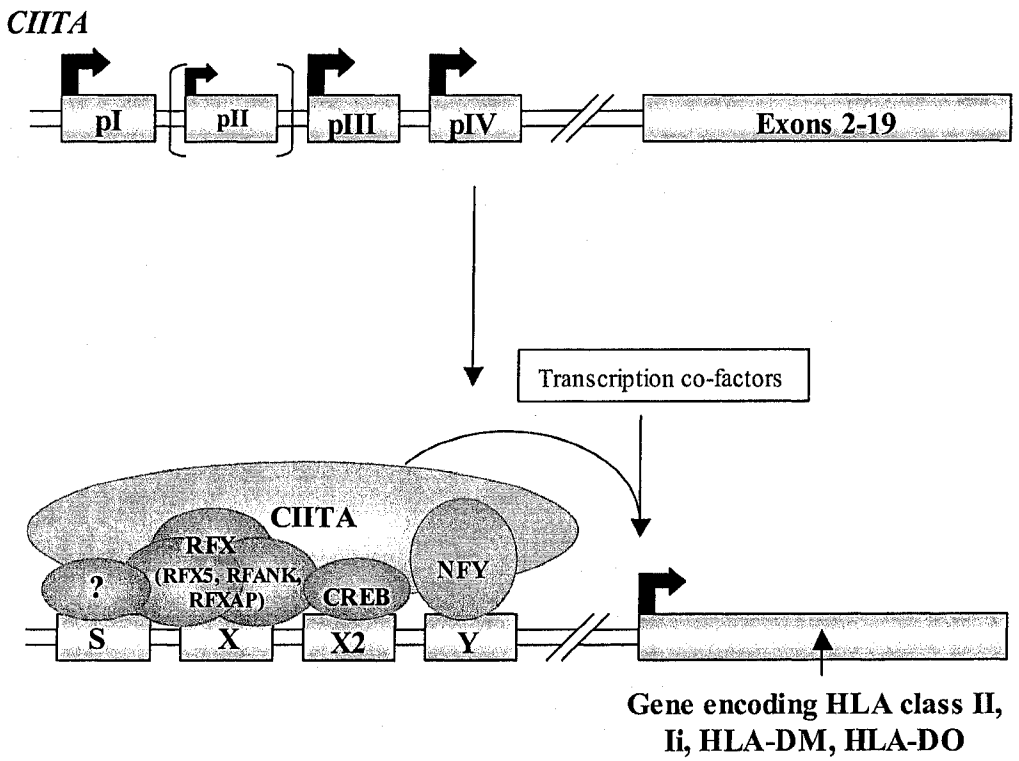


Figure 1.3: CIITA is the master regulator of HLA class II gene transcription. The human CIITA gene has four promoters (PI-IV) that control the transcription of four different first exons connected to a common second exon. PI, PIII and PIV control CIITA transcription in myeloid DC and macrophages, B cells and plasmacytoid DC, and IFN-γ induced CIITA, respectively. The function of PII is unknown. All HLA class II genes contain a SXY motif, comprised of S, X, X2 and Y boxes, which are bound cooperatively by the trimeric RFX, CREB, NFY and an unidentified S-box-binding protein. This four-protein complex forms the enhanceosome that binds CIITA, which drives the transcription HLA class II genes via recruitment of co-factors involved in histone acetylation, methylation, chromatin remodeling and transcription initiation and elongation. *Figure adapted from Reith, LeibundGut-Landmann and Waldburger (2005)* [99].

[106]. These mice illustrate the importance of CIITA-PIII for B cell MHC class II expression as they completely lack expression of CIITA and MHC class II in both B-1 and B-2 B cell subsets and fail to produce antibodies to the T-cell-dependent antigen (4-hydroxy-3-nitrophenyl)-acetyl-chicken gamma globulin. CIITA and MHC class II expression was also lacking in plasmacytoid DC, while expression was unaltered in myeloid DC and macrophages. In summary, constitutive CIITA expression is controlled by CIITA-PI in myeloid DC and macrophages and CIITA-PIII in B cells and plasmacytoid DC, while IFN-γ inducible CIITA in non-hematopoeitic cells is controlled by CIITA-PIV.

1.4.4.3 IFN-γ induction of CIITA-PIV

IFN-γ induces CIITA-PIV activation via its effects on three regulatory elements located proximal to CIITA-PIV, an IFN-γ-activated site (GAS), an E box and an IFN-regulatory factor element (IRF-E) (Figure 1.4). IFN-γ binding to the IFN-γ receptor induces Janus kinase (JAK)-1 and JAK-2 activation, which phosphorylate signal transducer and activator of transcription (STAT)-1. STAT-1 then dimerizes and translocates to the nucleus where it binds the GAS element, in concert with upstream transcription factor (USF)-1, which binds the E-box [107]. STAT-1 also induces IRF-1 activation, which then binds with IRF-2 to the IRF-E region of CIITA-PIV [108]. STAT-1, USF-1, IRF-1 and IRF-2 binding to regulatory elements in CIITA-PIV leads to transcription initiation and CIITA expression [99].

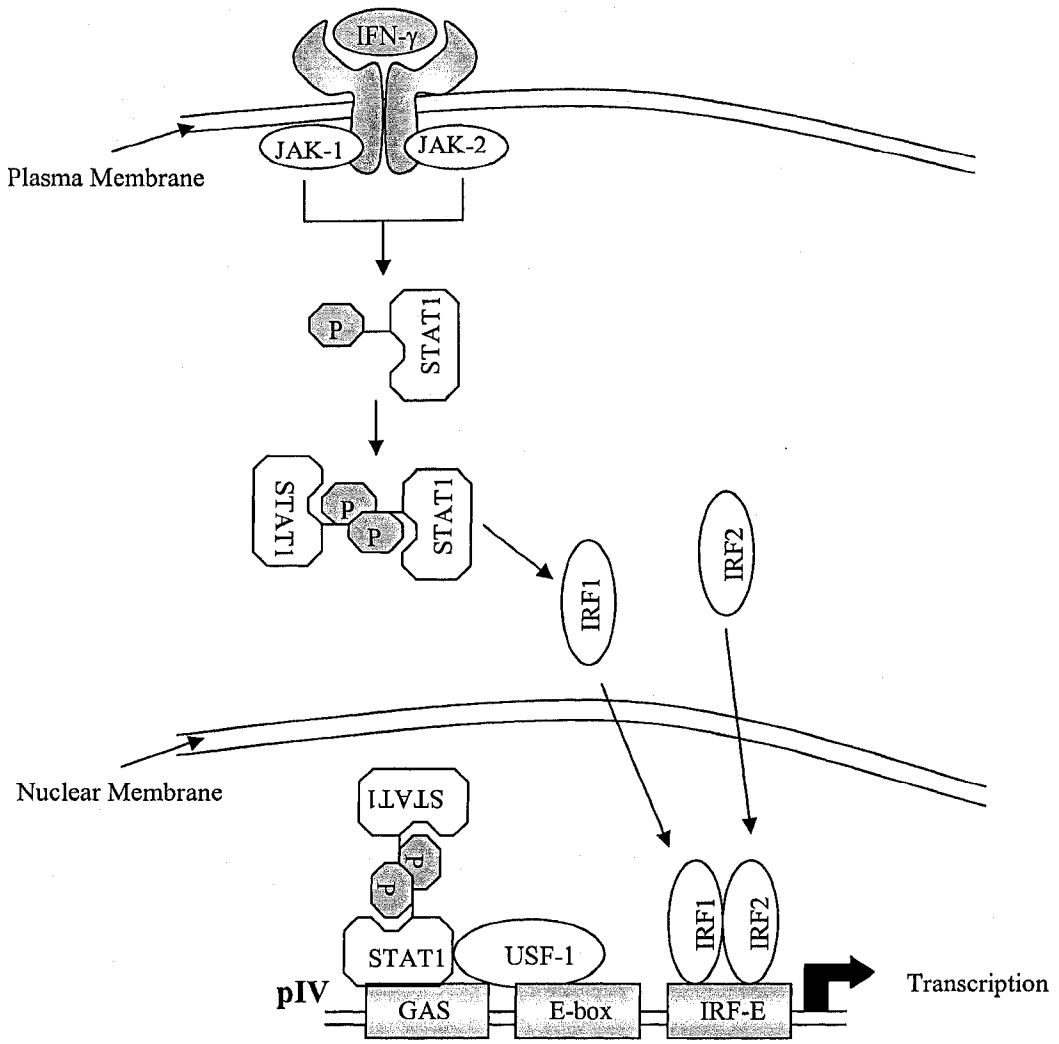


Figure 1.4: Induction of CIITA promoter IV (PIV) transcription via IFN-γ. Binding of IFN-γ to its receptor induces Janus kinase (JAK)-1 and JAK-2 cross-phosphorylation, which in turn phosphorylate signal transducer and activator of transcription (STAT)-1. Phosphorylated STAT-1 dimerizes, translocates to the nucleus and binds co-operatively with upstream transcription factor (USF)-1 to the IFN-γ-activated site (GAS)-E-box motif of PIV. STAT-1 also activates IFN-regulatory factor 1 (IRF1), which binds co-operatively with IRF2 to the IRF-element (IRF-E) of PIV. *Figure adapted from Reith, LeibundGut-Landmann and Waldburger (2005)* [99].

1.4.4.4 HLA-DRB Promoter Polymorphisms

Although HLA class II genes are generally coordinately regulated by CIITA, discordant expression has also been reported. For example, the RJ2.2.5 B cell line, which is transfected with a dominant negative mutant of CIITA, lacks expression of DRα, DRβ and DQα, while DQβ, DPα, DPβ, DOα, DOβ, DMα and DMβ are expressed in the absence of CIITA [109]. Furthermore, low levels of Ii and HLA-DM transcripts are found in the BLS-2 B cell line [110] and the CEM T lymphoblastoid cell line [111], which lack functional CIITA genes. Additional *cis*-acting elements have been identified in the promoters of Ii and HLA-DM which likely contribute to their expression in the absence of CIITA [112, 113].

Mitchison and Roes (2003) proposed that polymorphisms in the coding and regulatory sequences of MHC genes co-evolved in response to infection, following their discovery of linkage disequilibrium between murine MHC class II promoter variants and exon 2 sequences, which encode the peptide binding site of MHC class II molecules [114]. Polymorphisms within the promoters of HLA class II genes cause transcription factors to bind with varying affinities. Sequencing studies of the DRB promoters of Epstein-Barr virus (EBV)-transformed B cell lines (BCL) revealed nucleotide variability in the X and Y boxes and the spacers between the two as well as sequences containing CCAAT and TATA boxes [115, 116].

The influence of these promoter polymorphisms on the transcriptional activity of HLA-DRB genes was shown by promoter-chloramphenicol acetyltransferase (CAT) reporter assays performed in BCL. Louis et al. (1994) compared DRB promoters from all HLA-DR haplotype groups and showed the hierarchy of transcriptional activity was in

the order DRB1 and DRB3 alleles of the DR52 haplotype group > DRB1 alleles of DR1, DR8 and DR51 haplotype group > DRB1 and DRB4 alleles of the DR53 haplotype group. DRB5 showed weaker transcriptional activity than its associated DRB1 alleles. Nucleotide substitution studies showed X box polymorphism accounts for the observed transcriptional activity differences [117].

Vincent et al (1996) determined the steady state levels of DRB messenger ribonucleic acid (mRNA) in peripheral blood B cells using competitive RT-PCR and showed a similar hierarchy of transcriptional activity that correlated with the amount of transcribed mRNA for all except DRB1*04, which was over expressed as compared to the transcriptional activity of the DRB1*04 promoter, suggesting a post-transcriptional mechanism modulates HLA-DRB1*04 mRNA expression [118]. Further evidence for post-transcriptional control of HLA class II expression was provided by ribonuclease protection assay comparison of pre-mRNA and mRNA levels in a DRB1*07 homozygous BCL. Although DRB1*07 pre-mRNA was 3-4 times greater than DRB4 pre-mRNA, DRB1*07 mRNA was 7 times greater than DRB4 mRNA [119]. A similar assay, performed using PBMC from 7 healthy DRB1*07$^+$ donors, confirmed this difference for two DRB4 alleles and showed that DRB4*0101 is expressed at higher levels than DRB4*0103, when normalized to DRB1*07 pre-mRNA or mRNA from the same individual [120].

1.4.5 Influence of MHC class II polymorphisms on CD4$^+$ T cell responsiveness

Following recognition of the MHC-peptide complex via the T cell receptor (TCR), CD4+ T_H cells can become activated and differentiate into either T_H1 cells characterized by the secretion of IL-2 and IFN-γ or T_H2 cells characterized by the

secretion of interleukin (IL)-4 and IL-10. T_H1 cells are crucial for the development of cellular immunity and activation of CD8+ cytotoxic T lymphocytes. In contrast T_H2 cells function to elicit humoral immune responses through B cell activation and induction of antibody production. T_H cell differentiation is largely dependent on the influence of surrounding cytokines with IL-12 and IFN-γ enhancing T_H1 development, while IL-4 promotes T_H2 development, coincident with the interaction with MHC-peptide complexes [121].

The level of expression of MHC class II molecules influences T cell activation and ensuing cytokine production. For example, flow cytometry and bead quantification of $CD11b^+$ bone marrow derived macrophages from mice of different H-2 haplotypes revealed $I\text{-}A^b$ and $I\text{-}E^K$ molecules were expressed at higher levels and for a longer time period than $I\text{-}A^d$, $I\text{-}A^k$, and $I\text{-}A^q$. Immunization of mice with keyhole limpet hemocyanin (KLH) or chicken immunoglobulin, followed by *in* vitro re-stimulation of T cells isolated from the draining lymph node showed $I\text{-}A^b$-restricted T cells were more frequently $IFN\text{-}\gamma^+$ and $IL\text{-}4^-$, while $I\text{-}A^d$ restricted T cells were more frequently $IL\text{-}4^+$ and $IFN\text{-}\gamma^-$ [122]. Antigen dose also influences the T_H1/T_H2 polarization of naïve $CD4^+$ T cells, with low dose antigen favoring T_H2 polarization with high IL-4 production and high dose antigen favoring T_H1 polarization and IFN-γ production [123].

Polymorphisms in HLA class II alleles that affect the affinity of HLA for peptide also influence the cytokine production and proliferation of $CD4^+$ T cells. HLA-DR-restricted $CD4^+$ T cell lines specific for an immunodominant and promiscuously recognized peptide of the bacterium *Mycobacterium tuberculosis* all proliferated in a HLA-DR restricted manner in response to peptide pulsed autologous irradiated peripheral

blood mononuclear cells (PBMC). However, the greatest IFN-γ production, proliferative response and cytotoxicity were directed against HLA-DR-matched EBV-transformed homozygous BCL expressing either DRB1*0101, *1501 or *0401, which bound the peptide with the highest affinity (IC_{50} < 10 μM, as determined by competitive MHC binding assay). HLA-DR polymorphisms, located outside the peptide-binding groove in positions β180-189, also influence interaction with the CD4 co-receptor of the T-cell receptor (TCR) and resultant IL-2 production [124]. Experimental studies have also demonstrated high levels of MHC class II/CLIP complexes, formed from exogenously added CLIP or endogenously expressed by APC, modulated antigen-specific effector T cells, inducing a shift from T_H1 to T_H2 responses [125, 126].

T cell activation and cytokine production are also affected by the affinity of the peptide for TCR. Studies using altered peptide ligands revealed that varying the sequence of the peptide bound to TCR shifts T_H cell responses from IFN-γ producing T_H1 cells to IL-4 producing T_H2 cells. This skewing was attributed to decreased signal strength through the TCR by the altered peptide ligand, resulting in decreased zeta-chain-associated protein kinase (ZAP)-70 phoshorylation and decreased calcium flux [127].

1.5 HLA class II allele associations with breast carcinoma

Studies of HLA class II associations with disease susceptibility and prognosis and outcome in cancer are complicated by the enormous genetic variation of the HLA system. As a consequence of this complexity, several contradictory reports have arisen from such studies. For example, early serological studies found HLA-DR4 was increased in Russian breast carcinoma patients (46.8%) as compared to the general Moscow population (16.3%). In addition, HLA-DR4 positive patients more frequently had ER^- tumors and

had a worse outcome (50% increase in the 5 year survival of the DR4 negatives compared with the DR4 positives) [128]. In contrast, the frequency of HLA-DR4 was decreased in Italian women with breast cancer (2/62, 3.2%) as compared to controls (29/242, 12%) [129]. Another serological study revealed HLA-DRw6 (DRB1*13 and DRB1*14) was increased in Chinese breast cancer patients and associated with ER expression [130].

The first molecular-based investigation of HLA class II alleles and disease susceptibility in breast cancer was performed by Chaudhuri et al. (2000) [131]. Using PCR with sequence-specific primers (PCR-SSP) to DNA-type early-onset breast cancer patients for HLA-DR, -DP and -DQ alleles, they found DQB1*03032 and DRB1*11 were significantly decreased in patients. In contrast, Gourley et al (2003) did not reveal a protective effect of DRB1*11 [132]. These differences may reflect study design as Gourley et al. (2003) did not limit patient selection to early age of onset. Another small molecular typing study of 36 Iranian breast cancer patients revealed a significant association of DRB1*12 and disease susceptibility [133]. These molecular-typing studies have focused on disease association and were not designed to evaluate associations of HLA class II alleles with prognostic parameters and outcome in breast carcinoma patients.

1.6 Expression of HLA class II antigens in breast carcinoma

1.6.1 Expression of HLA class II in breast cancer cell lines

In contrast to HLA class I molecules, HLA class II expression is typically restricted to APC, such as B-cells, macrophages and dendritic cells. However, HLA class

II can be induced on other cell types, including breast cancer cell lines, by IFN-γ [134], IL-1α, IL-1β [135], TNF-α [136] and IL-4 [137]. Several groups have examined IFN-γ up-regulation of HLA-DR on breast cancer cell lines using immunofluorescence and flow cytometry [134, 136, 138]. These studies employed different anti-HLA-DR monoclonal antibodies (L243, Bra30, and D1-12) but all found that HLA-DR antigens were induced after IFN-γ treatment. The same result was obtained for several of the cell lines using radioimmunoassay to detect HLA-DR expression [64, 139]. A more recent study, in our laboratory, found constitutive and IFN-γ inducible expression of HLA class II and the co-chaperones Ii and HLA-DM in breast carcinoma cell lines (BCCL). HLA-DR, HLA-DP, Ii and HLA-DM were constitutively expressed in 4/11, 2/11, 4/11 and 2/11 BCCL and inducible in 7/11, 8/11, 7/11 and 5/11, respectively. None of the BCCL constitutively expressed HLA-DQ, but it was inducible in 5/11 BCCL. HLA-DRβ allotypic expression was also examined in these cell lines and 5/11 showed selective up regulation of DRβ allotypes by IFN-γ [140].

1.6.2 Expression of HLA class II in breast tissue

1.6.2.1 Classical HLA class II expression in breast carcinoma

HLA class II molecules are not expressed by resting breast epithelial cells but are present *de novo* in the lactating breast [141, 142], likely up-regulated in response to prolactin [143]. As breast tumors are frequently infiltrated by immune cells [144], it is plausible that HLA class II molecules may be up-regulated on tumor cells in response to the cytokine milieu of the surrounding tumor microenvironment. Alternatively, HLA class II expression on breast tumor cells may be independent of cytokine modulation and

due to molecular changes induced during the progression to carcinoma as several groups have shown HLA class II expression in benign hyperplastic breast epithelium [141, 145-148]. However, the presence of lymphocytic infiltrating cells adjacent to HLA-DR expressing non-neoplastic epithelial cells [146] suggests cytokines produced by stromal inflammatory cells may also modulate HLA class II expression in benign breast epithelium.

To date, IHC staining of over 1000 surgically removed breast carcinoma lesions has shown that HLA class II antigens are often up-regulated on malignant tumors of mammary epithelial cells [141, 145-158]. The frequency of HLA class II antigen expression in breast carcinoma lesions has been reported to average around 30%, however, frequencies have varied between 13% (10/77) [152] and 89% [150]. The differences among the reported frequencies of HLA class II antigen expression in breast carcinoma lesions are likely to result from the effect of many variables including patient population characteristics, sample size, criteria utilized to score HLA class II antigen expression and methodological differences in fixation protocols and antibodies used. All studies investigated HLA-DR antigen expression while only five have examined HLA-DP and HLA-DQ antigen expression. In all five studies, irrespective of the frequency of HLA class II antigen detection in the breast carcinoma lesions analyzed, HLA-DR antigens were more frequently expressed than HLA-DQ and DP antigens [146, 147, 151, 156, 159].

1.6.2.2 Expression of HLA class II co-chaperone molecules in breast carcinoma

No studies have reported on the expression of HLA-DM in breast carcinoma while two studies examined Ii expression [146, 151]. Both reported Ii is expressed in a

greater subset of breast carcinomas than HLA-DR, followed by HLA-DP and HLA-DQ, yet the implications of this discordant expression of Ii and HLA-DR in breast carcinoma remain unclear. Ii is expressed at higher levels than HLA-DR in invasive colon carcinoma and while high Ii expression associated with poorly differentiated tumors, no relationship with tumor stage was observed [160]. In contrast, Ii expression by gastric tumor cells did not associate with tumor grade but was increased according to depth of invasion and also associated with decreased survival [161]. These studies did not address the discordant expression of Ii and HLA-DR but merely related both factors independently with prognostic factors. If not fortuitous, the association of Ii expression with disease progression may be due to the prevention of tumor antigen (TA)-derived peptide presentation to $CD4^+$ T cells for recognition, since *in vitro* studies have shown that HLA class II-transfected tumor cells are unable to present TA-derived peptides to $CD4^+$ T cells in the presence of Ii [89, 138].

1.7 The biological and prognostic significance of HLA class II antigen expression in breast carcinoma

1.7.1 Associations of tumor cell HLA class II expression with T cell infiltration in breast carcinoma

Working on the hypothesis that the induction of HLA class II antigens on breast cancer cells is attributable to the cytokine milieu within the tumor, which is largely influenced by cytokine producing infiltrating T cells, one would expect to see a clear association between the two factors. Yet, previous studies have not identified a clear relationship between the two as some groups have reported a positive relationship [162], while others showed no association [145, 154]. Some of the discrepancies in these studies

may be dependent on the subjective and inconsistent scoring schemes for tumor cell HLA expression and infiltrating T cells.

The lack of association of tumor cell HLA class II expression and infiltrating T cells may reflect the absence of co-stimulatory molecule expression by tumor cells, resulting in T cell anergy [163]. However, a study conducted in our laboratory revealed although BCCL lack expression of the co-stimulatory molecules CD80 and CD86, the co-stimulatory molecule CD40 was expressed in 7/11 BCCL [140]. Thus, additional co-stimulatory molecules expressed on breast tumor cells may provide the second signal necessary for T cell activation in the context of breast carcinoma. In particular, in the context of anti-tumor immunity, initial naïve T cell activation is likely via DC, which present tumor antigen derived peptides in the draining lymph node and such antigen-specific T cells have less stringent requirements for subsequent re-activation at the tumor site [164].

The association of HLA class II expression by tumor cells and infiltrating T cells is likely dependent on numerous factors including co-stimulatory molecule expression, the phenotype of infiltrating T cells (and subsequent cytokine production) and the intratumoral pattern of T cell distribution. However, there is substantial evidence to support a role for tumor cell expression of HLA class II antigens in the induction of anti-tumor immunity (discussed in the following section).

1.7.2 Experimental evidence to support a role for tumor cell HLA class II antigen expression in anti-tumor immunity in breast carcinoma

While the importance of $CD8^+$ T cells in the anti-tumor immune response has long been recognized, several articles have also highlighted the requirement for $CD4^+$ T

cell-recognition of tumor peptides presented by HLA class II molecules (HLA-DR, DP, DQ) for optimal anti-tumor immunity [165-167]. While initial CD4+ T_H cell activation occurs in the tumor draining lymph node via DCs that have obtained tumor associated antigens (TAA) at the tumor site, experimental evidence from murine studies has identified a role for HLA class II antigen expressing tumor cells in the generation of effective anti-tumor immunity. Ostrand-Rosenberg's group, have shown that the HLA class II-transfected murine SAI sarcoma cell line efficiently presents tumor peptides and transplantation of these tumor cells into syngeneic mice results in the generation of a $CD4^+$ anti-tumor immune response and elimination of the tumor [89, 138]. Recently, the importance of HLA class II antigen expression in breast carcinoma was shown by transfecting a murine mammary adenocarcinoma cell line with CIITA. This resulted in expression of HLA class II antigens and rejection of the transplanted tumor in syngeneic mice in a $CD4^+$ T cell dependent manner [168].

Human studies have identified HLA class II restricted tumor antigen-specific $CD4^+$ T cells exist in the peripheral blood and tumor infiltrating lymphocytes (TIL) of breast cancer patients and have shown that breast tumor cells can function as APC. For example, Tuttle et al (1998) [169] showed that peripheral blood $CD4^+$ T cells from primary breast cancer patients with HER-2/neu positive tumors proliferated *in vitro* in response to primary stimulation of PBMCs with HER-2/neu derived peptides. Furthermore, direct recognition of class II positive breast tumor cells was shown by Dadmarz et al (1995) [170] who found that cultured $CD4^+$ TIL produced granulocyte-macrophage stimulatory factor (GM-CSF) and TNF-α, in a HLA-DR-restricted manner, in response to stimulation with autologous tumor cells. Sotiriadou et al (2001) [171]

showed that the HER-2/neu tumor antigen was a naturally processed and presented tumor antigen as $CD4^+$ T cell clones, derived from a healthy volunteer, proliferated and produced IFN-γ in response to the HER-2/neu^+ breast cancer cell line SKBR3 induced to express HLA class II antigens by combined IFN-γ and TNF-α treatment. Antibody blocking experiments showed that this recognition was HLA-DR restricted. Perez et al (2002) further showed that HER-2/neu was naturally processed and presented by HLA-DR antigen expressing breast, colorectal and pancreatic tumor cells, which were directly recognized by Her-2/neu-specific $CD4^+$ T cell clones derived from a healthy individual [172].

1.7.3 Associations of tumor cell HLA class II expression with prognosis and outcome in breast carcinoma

The finding of HLA class II antigen expression in benign breast lesions and well-differentiated tumor cells with apparent difference in inflammatory infiltrate between HLA-DR antigen positive and negative tumors, led some to hypothesize that HLA class II antigen expression is not influenced by cytokines but rather by the differentiation state of the tumor [147]. Two other studies observed a relationship between tumor cell expression of HLA-DR antigens and low tumor grade [153, 159]. However, four other studies have found no significant associations of HLA class II antigen expression and the differentiation state of the tumor [146, 150, 155, 162].

The histological type of the tumor may also affect HLA class II expression by tumor cells. One study comparing HLA-DR antigen expression in different tumor types found DR expression is much higher in medullary, than atypical medullary or invasive

ductal breast carcinomas [157]. However, others have found no difference in HLA class II antigen expression across tumor types [146].

A role for hormonal control of HLA class II antigen expression was suggested by Brunner et al. (1991) who found HLA-DR antigen expression was significantly associated with estrogen and progesterone receptor expression [159]. No such relationship was observed in three other studies [150, 152, 155]. HLA class II antigen expression in primary tumor lesions has not been found to associate with lymph node metastasis [154, 156] tumor stage [152, 159] or mitotic index [154, 155]. Likewise, HLA class II expression is not an independent predictor of either disease-free or overall survival [152, 155].

The discrepancies in the aforementioned studies may reflect methodological variability and are also likely to be influenced by the complexity of breast carcinoma, which is not a single disease entity, and is characterized by many different interacting factors. For example, women with the most common histological type of breast carcinoma, invasive ductal, are more likely to be diagnosed at an earlier age and with tumors that are poorly differentiated, hormone receptor negative and of smaller diameter, whereas women with the next most common type, invasive lobular breast carcinoma, are more frequently diagnosed at a later age and with tumors that are better differentiated, of larger diameter and more often hormone receptor positive [173]. Thus, the interplay of these factors combined with hormones and cytokines in the tumor microenvironment is likely to influence up-regulation of HLA class II antigen expression by tumor cells.

1.8 The immune response to breast carcinoma

1.8.1 Inflammatory cell infiltration in breast carcinoma

Mononuclear cell infiltration is markedly increased in invasive breast carcinoma as compared to non-malignant breast tissue [145, 162, 174]. Several studies in the 1980's and 1990's used immunohistochemistry or flow cytometry of collagenase disrupted tumors to characterize the immune cell infiltrate in breast carcinoma lesions at the time of surgery. T cells and macrophages were reported to be the major tumor infiltrating cell subsets with some studies reporting T cells exceed macrophages [162, 174-176] and others reporting the opposite [144, 177, 178]. Likewise, the $CD4^+/CD8^+$ T cell ratio has varied among studies with some groups finding the majority of tumors have greater numbers of $CD4^+$ T cells [145, 162, 176, 178, 179] and others reporting the opposite [175, 180, 181]. Dendritic cells (DC) are also abundant in breast carcinoma, however, mature DC are located at the tumor periphery while intratumoral DC have an immature phenotype [182]. The number of B lymphocytes, as detected by antibodies to CD19 or CD20, is reduced in comparison to T cells and macrophages [144, 162, 175, 176, 181, 183] and breast carcinomas contain a relative paucity of NK cells, determined using antibodies to CD16, CD56 and CD57 [174-176, 179, 183]. Tissue mast cells, plasma cells and eosinophils are also present in low numbers [178].

1.8.2 Central roles of $CD4^+$ T cells in the anti-tumor immune response

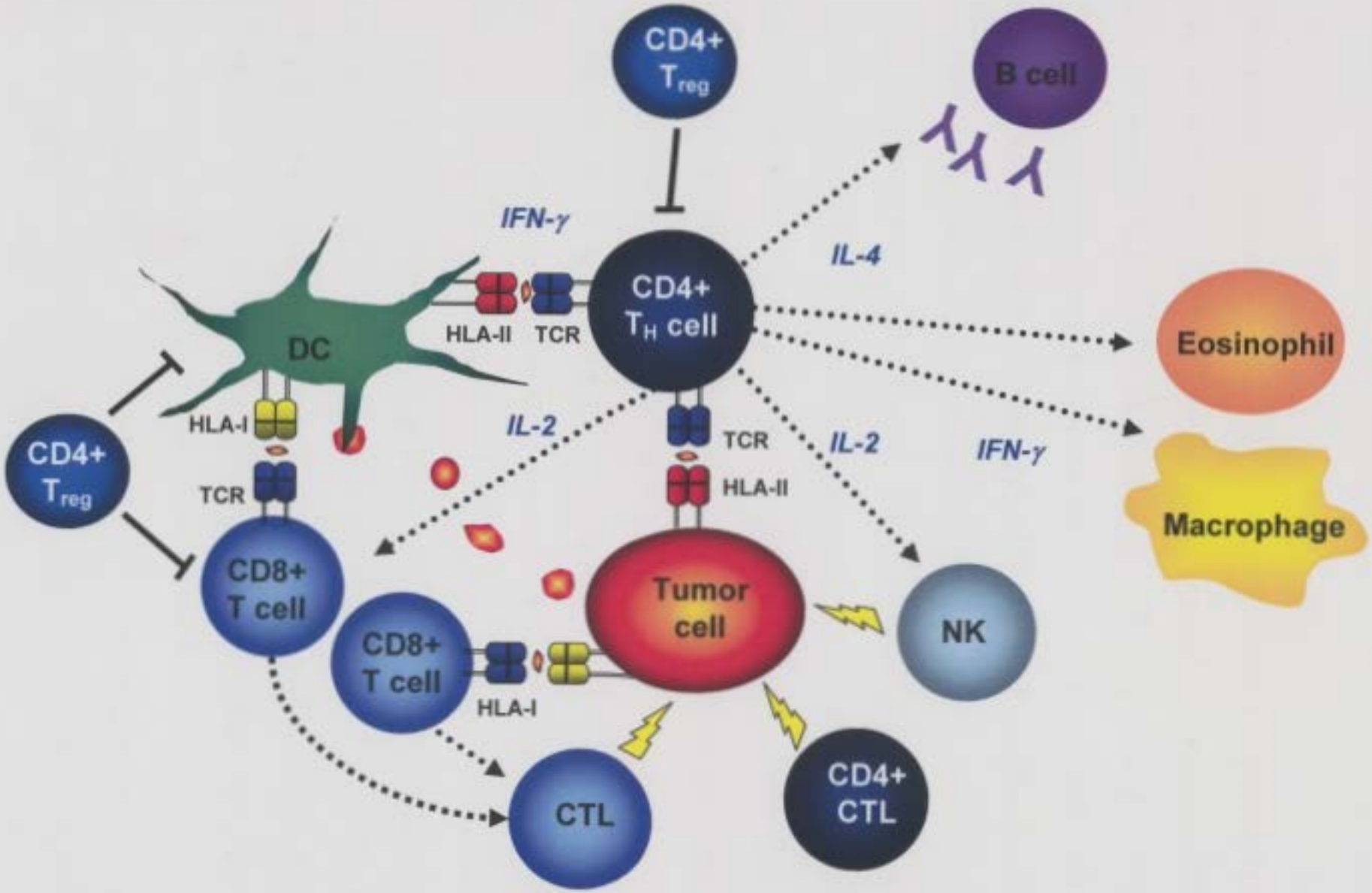
The importance of HLA class II molecules in the initiation of immunity is via the activation of CD4+ T_H cells, which recognize peptides in the context of HLA class II molecules. Experimental models have demonstrated CD4+ T_H cells are central to the

anti-tumor immune response as they function as 'helper' cells orchestrating multiple arms of the immune response through their activation of immune effector cytotoxic T lymphocytes (CTL) and B lymphocytes and their recruitment and activation of cells of the innate immune response such as macrophages, eosinophils and NK cells. Recent studies have identified a substantial proportion of CD4+ T cells infiltrating breast tumors are immunoregulatory, characterized by secretion of the immunosuppressive cytokines IL-10 and transforming growth factor (TGF)-β1 and capable of *in vitro* suppression of effector cell proliferation and cytokine secretion [184]. Such CD4+ T_{reg} cells can suppress anti-tumor immunity via their actions on DC, naïve CD4+ T_H cells and effector CD4+ and CD8+ T cells (Figure 1.5).

1.8.3 The prognostic significance of infiltrating $CD4^+$ T cells in breast carcinoma

While the importance of $CD4^+$ T cells in orchestrating the anti-tumor immune response in murine models is well established, the clinical significance of $CD4^+$ T cell infiltration in human breast carcinoma remains unclear. Many studies have attempted to correlate the degree of T cell infiltration with prognostic parameters. Infiltrating T cells negatively associate with LN metastasis is some studies [154, 185], while the opposite was reported in other studies [186, 187]. Contradictory reports regarding associations of infiltrating T cells and tumor stage have also arisen [181, 185]. Several groups have shown infiltrating T cells associate with the poor prognostic indicator of increased histological grade [162, 174, 187], while others found no association [179, 181]. Likewise, the majority have reported an inverse correlation between hormone receptor expression and T cell infiltration [174, 181, 187], yet others did not observe such an association [179, 186]. Despite published associations of infiltrating T cells with

Figure 1.5: Central roles of CD4+ TH cells in anti-tumor immunity. Tumor cells shed antigens, which are endocytosed by dendritic cells, processed and presented in the context of HLA class II to $CD4^+$ T_H cells and HLA class I to $CD8^+$ T cells, following dendritic cell (DC) migration to the draining lymph node. Activated $CD4^+$ T_H cells then traffic to the tumor where they orchestrate multiple arms of the anti-tumor immune response. $CD4^+$ T_H1 cells secrete IL-2 and IFN-γ and are crucial for the induction of cellular immunity. IL-2 secreted by $CD4^+$ T_H1 cells activates MHC class I-restricted $CD8^+$ T cells, driving their differentiation to cytotoxic T lymphocytes (CTL) and NK cells, which directly lyse HLA class I+ tumor cells. IFN-γ produced by T_H1 cells activates immature DC enabling their efficient presentation of tumor associated antigens to $CD8^+$ T cells and tumor associated macrophages (TAM), which lyse tumor cells via secretion of TNF-α and reactive oxygen and nitrogen species. $CD4^+$ T_H2 cells secrete IL-4, inducing B cell activation and differentiation into tumor-specific antibody secreting plasma cells and recruitment and activation of eosinophils, which degranulate. $CD4^+$ T cells can also be directly activated by and lyse HLA class II^+ tumor cells via secretion of tumoricidal cytokines. The anti-tumor immune response can be suppressed in the presence of immunoregulatory TAA-specific CD4+ T_{reg} cells via suppression of naïve CD4+ T_H cell proliferation, inhibition of cytokine secretion by CD4+ and CD8+ effector T cells and suppression of DC maturation. *Figure adapted from Goedegebuure and Eberlein (1995)* [186], *Pardoll and Topalian (1998)* [164], *Wang (2001)* [187] *and Wang and Wang (2005)* [188].


CD4+
T_{reg}
B cell
IFN-γ
IL-4
CD4+
T_H cell
DC
HLA-II
TCR
Eosinophil
HLA-I
IL-2
TCR
IL-2
IFN-γ
CD4+
T_{reg}
TCR
HLA-II
Macrophage
CD8+
T cell
Tumor
cell
NK
CD8+
T cell
HLA-I
CTL
CD4+
CTL

prognostic parameters, the degree of T cell infiltration fails to predict relapse-free or overall breast cancer survival [187, 191, 192]. A more recent study of ovarian carcinoma patients has outlined the importance of studying the distribution of T cells within the tumor as the presence of intratumoral T cells independently predicted improved disease-free and overall survival in ovarian cancer patients [193].

The discrepancies in the above studies may in part be due to the presence of regulatory T cell subsets. Not all $CD4^+$ T cells that infiltrate breast tumors function in an effector capacity. Breast cancer patients have increased numbers of functionally immunosuppressive CD4+CD25+ T_{reg} cells within the tumor microenvironment and circulation [184, 194]. Thus, the balance of effector and regulatory cell subsets will have important consequences for the anti-tumor immune response and prognosis in breast carcinoma patients. Indeed, a high CD8+/CD25+FOX-P3+ T_{reg} TIL ratio associates with improved survival in ovarian carcinoma [195].

1.8.4 The cytokine milieu in breast carcinoma

1.8.4.1 In situ cytokine production by tumor infiltrating immune cells

While it is well documented both CD4+ and CD8+ isolated TIL are capable of cytokine secretion in response to stimulation with autologous or HLA-matched allogeneic tumor cells [170, 188, 196] or non-specific mitogen stimulation *in vitro* [197], few studies have evaluated *in situ* cytokine production in breast carcinoma. A small-scale, *in situ* hybridization study of 13 breast carcinomas, using cytokine specific probes for IL-2, TNF-α and IFN-γ mRNA, revealed for the majority of tumors (9/13), few mononuclear cells express these cytokines. However, mucinous breast carcinomas (N=4) contained a

much higher percentage of cytokine producing cells, and it was suggested tumor-associated mucins may provide increased immunogenicity [198]. This study was limited in size and also failed to relate the number of cytokine producing cells with the degree of mononuclear cell infiltration. However, 7/9 tumors with low numbers of cytokine expressing cells had minimal mononuclear cell infiltration. Camp et al. (1996) used immunohistochemistry to assess protein expression of a much larger panel of cytokines in 89 primary breast carcinomas and 14 benign breast lesions [191]. Significantly more tumors contain tumor infiltrating T cells that produce IL-2, IL-4, TGF-β1 and TNF-α than IFN-γ and GM-CSF, using a cutoff of 3% positive TIL. Furthermore, IL-4, IL-2 and TGF-β1 production was significantly higher in breast carcinoma as compared to benign breast tumors. However, this study did not dual stain infiltrating T cells but rather compared cytokine stained sections to CD3 stained sections to ascertain T cell specific cytokine production.

The relationship of *in situ* cytokine production with prognostic parameters was investigated in a small study, of 19 breast carcinoma and 7 benign breast lesions, where cytokine mRNA was detected using RT-PCR and Southern hybridization of bulk tissue RNA [183]. The majority of tumors expressed TNF-α (12/19), TGF-β1 (12/19) and IL-10 (13/19). IL-4, IL-6, and IFN-γ were not detected but a subset expressed IL-2 (7/19) and IL-8 (2/19) mRNA. Only IL-2 (2/7) and IL-10 (1/7) were detected in benign lesions. The presence of detectable TNF-α did not associate with prognostic parameters but detectable IL-10 associated with increased tumor size. Relative TGF-β1 levels were semi-quantified using competitive PCR and were significantly decreased in tumors from patients of advanced stage as compared to those alive without residual disease. However, the true

significance of these results must be interpreted with caution due to the small sample size. Furthermore, despite knowledge of numbers of infiltrating cell subsets in 14/19 tumors, the authors failed to correlate cytokine mRNA with infiltrating cell subsets. Green et al. (1997) compared bulk tumor cytokine mRNA in a larger number of malignant breast tissues (N=77) to normal breast tissue (N=58). Relative amounts of mRNA were not determined but rather presence or absence of detectable mRNA was assessed. The proportion of tissues with detectable IL-1α, IL-1β, IL-4, IL-6, TNF-α and TNF-β did not significantly differ between the two groups, but IL-8 was more frequently detected in malignant tissues. IL-2, IL-3, IL-5, IL-7 and IFN-γ were not detected in either tissue. The expression of individual cytokine mRNA did not correlate with tumor stage, tumor grade, LN metastasis or menopausal status [199]. However, this study assessed cytokine mRNA in the absence of information on tumor infiltration by inflammatory cells.

1.8.4.2 Genetic polymorphisms that regulate cytokine production

Although it is likely the T cell mediated cytokine response in breast carcinoma will be dependent on the type and dose of tumor-associated antigens and the HLA allotypes expressed within the tumor, genetic differences in cytokine genes that influence cytokine production may also affect anti-tumor immunity. Cytokine and cytokine receptor genes generally have highly conserved exon sequences but often contain polymorphisms in regulatory regions [200]. Studies of cell lines transfected with cytokine allele promoter reporter gene constructs have shown single nucleotide polymorphisms (SNPs) affect cytokine gene transcription [201, 202]. Such polymorphisms also associate with differential cytokine production following *in vitro* activation of peripheral blood

mononuclear cells with mitogen or αCD3/αCD28 [203-206], and *in vivo* plasma levels from healthy blood donors [207, 208] and in response to vaccination [209].

Weak associations of SNPs in cytokine genes have been linked to prognosis and outcome and susceptibility or protection from breast cancer [210-216]. Contradictory reports have arisen with respect to such associations. For example, in a large prospective cohort study of 3075 American Caucasian women, the TGF-β1 Codon10 C/C genotype associated with decreased risk of breast carcinoma [217], while a large case-control study of UK, German and Finnish individuals did not demonstrate such an association [201]. The authors suggest this discrepancy might be explained by the increased number of cases (3987 patients and 3867 age-matched controls), decreased age and lack of non-invasive cases in their study. Discrepant reports also exist regarding associations of SNP genotypes with prognosis and outcome. For example, the IL-6 –174C allelic variant associated with LN metastasis, increased tumor grade and decreased overall survival of unselected breast carcinoma patients [218]. In contrast, in high-risk LN positive patients, the IL-6 -174C SNP associates with increased survival [219]. Importantly, studies of cytokine SNPs with prognosis and survival in breast carcinoma have not evaluated intratumoral cytokine production. Rather, such studies have grouped patients into high or low cytokine producing groups based on published reports of *in vitro* cytokine production. Yet, discrepant reports exist regarding associations of cytokine SNPs with *in vitro* cytokine production. It is important to note, studies aimed at investigating such associations have varied in their use of cell subsets, method and length of cell stimulation. For example, stimulation of PBMC from healthy individuals with Concanavalin A (ConA) or phytohemagglutinin (PHA) resulted in significantly increased

IFN-γ secretion from individuals homozygous for the +874T allele [205], while anti-CD2 stimulation of PBMC from healthy individuals did not demonstrate such an association [220]. Variation in the dose of stimulus can also influence associations as *in vitro* TNF-α secretion is significantly increased from individuals carrying the -308A allelic variant, following prolonged low dose lipopolysaccharide (LPS) stimulation (1ng/ml for 24hours) of whole blood, but not short-term high dose (100ng/ml for 3hr) treatment [221]. Kroeger et al. (2000) showed, using reporter gene assays, that the transcriptional activity of TNF-α promoter variants was influenced by both cell type and type of stimulation, suggesting cell-type specific transcription factors likely influence cytokine gene transcriptional control [222]. Furthermore, associations of cytokine gene polymorphisms with *in vitro* cytokine production, observed in healthy individuals are not always shared by individuals in a diseased state [223]. Assumptions should not be made regarding *in vivo* cytokine levels based on carriage of particular cytokine gene polymorphisms, as levels are surely dependent on the pattern of immune cell infiltration and the stimulus. Also, the multiple facets of anti-tumor immunity suggest patient outcome will be dependent on the balance of pro-inflammatory and immunosuppressive cytokines. Thus, associations of individual cytokine allelic variants with susceptibility and prognosis in breast carcinoma will likely be influenced by the other cytokine alleles carried by the patient.

1.9 OVERVIEW

1.9.1 Rationale and Thesis Overview

The preceding literature review has highlighted the fact that studies aimed at investigating associations of tumor cell expression of HLA class II antigens and the immune response and prognosis in breast carcinoma have been fraught with controversy.

Such studies have not assessed individual HLA class II allotypic expression but rather have examined HLA-DR, -DP and -DQ antigen expression, utilizing locus specific monoclonal antibodies. This may in part be due to the lack of availability of allospecific mAb that recognize determinants expressed in formalin-fixed and paraffin-embedded tissue sections. As allelic differences in HLA class II antigens are known to influence T cell activation and cytokine production, knowledge of tumor cell HLA class II expression in the absence of information on the HLA class II alleles carried by the tumor may be uninformative.

Based on the knowledge that allelic differences in HLA-DR molecules influence level of expression and T cell activation, we hypothesized that associations of tumor cell HLA-DR expression with T cell infiltration in breast carcinoma were influenced by the DRB alleles carried by the patient. Furthermore, as loss of expression of individual HLA class I allotypes was a common occurrence in breast carcinoma [224, 225] and experimental evidence suggests a role for T cell pressure in the generation of HLA loss variants [226, 227], we hypothesized that similar pressure from CD4+ T_H cells might exist and that individual HLA-DRβ allotypes would be differentially expressed by breast carcinoma cells. Due to the published association of HLA-DRB1*04 with a poor prognosis in breast carcinoma [128], we selected an equal number of tumors from HLA-DRB1*04+ and non-HLA-DRB1*04 patients to investigate these theories (Chapter 2).

As the study described in Chapter 2 was not designed to assess the clinical significance of HLA-DRβ allotypic expression in breast carcinoma (due to the biased selection for DRB1*04+ tumors, and the small sample size), we performed a larger study on a randomly selected set of breast carcinomas. We postulated that differential

expression of individual HLA-DRβ allotypes by breast tumor cells might represent an additional immune evasion strategy and thereby correlate with a poor prognosis. Furthermore, as HLA-DRB allelic variants influence immune responsiveness, we postulated that expression of particular HLA-DRβ allotypes might associate with a particular cytokine profile and with prognosis in breast carcinoma (Chapter 3).

Although murine studies have illustrated HLA class II expressing tumors cells can act as antigen presenting cells leading to $CD4^+$ T cell activation and tumor rejection, studies of human breast carcinoma have failed to investigate the *in situ* expression of the antigen processing machinery involved in the HLA class II pathway and their influence on immune responsiveness and patient outcome. Induction of HLA class II antigens and their related co-chaperones in breast carcinoma cells *in situ* is likely dependent on the cytokine milieu of the tumor microenvironment. We hypothesized that expression of components of the HLA class II antigen processing machinery by breast tumor cells would be influenced by the presence of infiltrating inflammatory cells and the *in situ* cytokine milieu. Furthermore, we anticipated expression of co-chaperone molecules would enable breast cancer cells to function as efficient APC and thereby correlate with improved patient outcome. To test these hypotheses, we evaluated tumor cell expression of HLA-DR and the co-chaperones Ii and HLA-DM and assessed the relationship of these factors to immune cell infiltrate and relative cytokine levels in a subset of breast carcinoma patients with prognostic and outcome information (Chapter 4).

As immune cells mediate their functions through the release of cytokines, we hypothesized the cytokine milieu of the tumor microenvironment would be dependent on the pattern of inflammatory cell infiltration and that published disparities in the

prognostic significance of inflammatory cell infiltration in breast carcinoma might be explained by variability in the balance of immune promoting and immune suppressing cytokines. Using our subset of invasive breast carcinoma patients with prognostic and outcome information, we assessed the relationships of inflammatory cell subset infiltration, cytokine mRNA levels and prognosis and outcome (Chapter 5).

Coincident with this, we further hypothesized some of the variability in intratumoral cytokine production might be dependent on genetic variability in cytokine genes, which have been documented to influence cytokine gene activity and secretion. To investigate this, we evaluated single nucleotide polymorphisms of a panel of cytokine genes and assessed the relationship to tumor derived cytokine levels, in a subgroup of our invasive breast carcinoma samples with prognostic and outcome information (Chapter 6).

The aim of this research project was to provide a comprehensive study, which takes into account the interplay of immunologically relevant markers on breast carcinoma cells (i.e., HLA-DR and the co-chaperones Ii and HLA-DM) with infiltrating inflammatory cells and immunomodulatory cytokine mRNA levels, in the context of knowledge of the HLA-DRB genetic differences of the patients. Breast carcinoma samples were phenotyped for the aforementioned immunological markers and a thorough statistical analysis was performed to assess the strength of relationship of these factors and their prognostic significance in the clinical course of breast carcinoma.

1.9.2 Co-authorship statement

The thesis author and S.D. were responsible for study design. All experiments were conducted under the supervision of S.D. The thesis author solely conducted all research and development studies to establish optimal immunohistochemistry (IHC)

protocols (as described in Chapter 2, Material and Methods), performed all IHC experiments (Chapters 2-6) and cytokine SNP genotyping experiments (Chapter 6) and contributed to the HLA class II DNA-typing of breast carcinoma samples described in Chapter 3. The thesis author was solely responsible for database construction, all statistical analysis and manuscript preparation, with major editorial contributions by S.D. Additional co-authors include J.D.R. (Chapters 2 and 4) who served as a second reader for the interpretation of all IHC slides and offered expert pathological expertise; D.C. who contributed to HLA class II DNA typing (Chapter 3) and conducted RT-PCR experiments (Chapters 3-6); P.W. (Chapters 2 and 4) of the Manitoba Breast Tumor Bank (MBTB) who provided the breast tumors utilized in these studies; and V.G. (Chapters 3 and 4) who provided statistical expertise.

CHAPTER 2: HLA-DRB ALLELES ARE DIFFERENTIALLY EXPRESSED BY TUMOR CELLS IN BREAST CARCINOMA[‡]

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2.1 ABSTRACT

The biologic and prognostic significance of HLA-DR expression and T-cell infiltration in breast carcinoma are presently controversial. To test the hypothesis that these factors are influenced by particular HLA-DRB alleles, 52 breast tumor samples, composed of 26 DRB1*04 and 26 non-DRB1*04 tumors, were assessed using immunohistochemistry for expression of HLA-DR and its associated invariant chain (Ii) and for infiltrating CD3+ T cells. While HLA-DR expression by tumor cells was significantly associated with T-cell infiltration, DRB1*04 tumors were more frequently HLA-DR+Ii+ and contained smaller CD3+ infiltrates than non-DRB1*04 tumors. This difference was largely attributable to DRB1*07 tumors, which were typically DR-Ii-, although they contained similar numbers of T cells to DR+Ii+ tumors. Further analysis of HLA-DR+ tumors using allotype discriminating antibodies revealed that DRB1*04 alleles were always expressed, while non-DRB1*04 alleles were inconsistently expressed. The results of this study provide the first reported evidence that HLA-DRB alleles influence HLA-DR expression and T-cell infiltration in breast carcinoma and suggest that multiple factors contribute to HLA-DR expression. Ongoing studies aimed at elucidating the molecular and immunologic mechanisms controlling differential HLA-DR expression and implications for prognosis and outcome should further our understanding of the antitumor immune response and evasion strategies employed by tumor cells

2.2 INTRODUCTION

A prerequisite for tumor eradication by CD8+ cytolytic T cells is recognition of tumor antigen presented by HLA class I molecules on the tumor cells and this is reflected by the numerous studies showing loss or down regulation of HLA class I on established

tumors [228, 229]. Optimal anti-tumor immunity also requires participation of CD4+ T cells, which recognize tumor peptides presented by HLA class II molecules (HLA-DR, -DP, -DQ) [138, 166, 167]. However, the importance of HLA class II expression on breast carcinoma cells and implications for anti-tumor immunity are currently unclear. Unlike HLA class I, HLA class II molecules are not normally present on resting epithelial cells, although they are detected *de novo* in the lactating breast and in a subset of breast carcinomas [141]. They can also be induced *in vitro* on many cell types, including breast cancer cell lines, by interferon-γ (IFN-γ) [134, 230] and other immunomodulators [136, 143], suggesting that *in situ* expression on carcinoma cells may be regulated by cytokines and/or hormones. Antigen presentation by HLA class II positive breast cancer cells, which lack co-stimulatory molecules typically found on professional antigen presenting cells (APC), could potentially induce T cell anergy. However, reports of direct recognition of tumor cells by HLA class II-restricted CD4+ T cells [170, 172] suggest that HLA class II+ tumor cells play an important role in determining the outcome of an anti-tumor immune response.

Studies investigating expression of HLA class II in breast carcinoma generally concur that HLA-DR and its co-chaperone Ii are more often and more strongly up regulated than HLA-DP or DQ [146, 151]. However, the significance of these findings is controversial as some studies suggested HLA-DR expression on breast tumor cells is associated with favorable prognostic indicators such as well differentiated tumors [147, 148] and hormone receptor expression [159], while others found no effect [146, 152]. Similarly, tumor cell HLA-DR was positively associated with T cell infiltration in some studies [162, 177] and unrelated in others [145, 147].

Interpretation of the above studies may have been hampered by the fact that generic and not allelic HLA-DR expression was examined. This was likely because of the paucity of allotype specific antibodies and the large number of HLA-DRB1 alleles, most of which are in linkage with additional structural HLA-DRB genes. These include HLA-DRB1*15 and 16, expressed in association with HLA-DRB5 (DR51); HLA-DRB1*03, 11, 12, 13 and 14 with HLA-DRB3 (DR52); HLA-DRB1*04, 07 and 09 with HLA-DRB4 (DR53); and HLA-DRB*01, 10 and 08 which are not in association with any other HLA-DRB expressed genes. As most individuals are heterozygous, expressing up to four HLA-DR allotypes and since carcinomas are known to selectively down regulate HLA class I alleles [228, 229], we hypothesized that HLA-DRB alleles would also be differentially expressed in HLA-DR+ tumors.

To test this hypothesis we compared DRB1*04 to non-DRB1*04 tumors. DRB1*04 has been reported to confer a poor prognosis in different carcinomas including breast [128, 231], but a similar study in our laboratory has not confirmed the association of DRB1*04 per se with poor prognostic factors in breast carcinoma patients. However, DRB1*04 allelic differences were noted as DRB1*0401 was increased in Stage I invasive ductal breast carcinomas, while other DRB1*04 alleles were increased in stage III tumors (data not shown). As part of this investigation, we also examined whether DRB1*04 and/or its individual alleles, influenced expression of generic HLA-DR, its co-chaperone Ii and T cell infiltration in breast carcinoma. HLA-DR+ tumors were further evaluated for expression of individual HLA-DRB alleles. Since HLA class I is ubiquitously expressed, but frequently down regulated on tumor cells, its expression was also assessed partly as an intrinsic control but also as an indicator of MHC integrity on the tumor cells.

2.3 MATERIAL AND METHODS

2.3.1 Patient Sample

Fifty-two invasive breast carcinoma samples were obtained from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The samples were comprised of 40 ductal, 6 lobular and 6 ductal-lobular mixed. The age at diagnosis ranged from 31 to 92 years (median 66 yrs). The tumors spanned a wide range of grade (grades 5–9), determined using the Nottingham grading system. Estrogen and progesterone receptor levels were determined by ligand binding assay and values ranged from 0 to 273 fmol/mg (median 21.5 fmol/mg) and 0 to 1088 fmol/mg (median 16.1 fmol/mg), respectively. Tumor size was available for 50 tumors and 36 had a diameter larger than 2 cm. Lymph node status was available for 43 patients of which 20 were positive.

The above cohort of 52 tumors were randomly selected from a larger sample that was DNA typed for HLA-DR by PCR-SSP using HLA class II typing kits (Bio-Synthesis, Inc., Lewisville, TX, USA) as part of an ongoing study in our laboratory (data not shown). To determine whether DRB1*04 alleles influenced HLA-DR or Ii expression by carcinoma cells or T cell infiltration, we investigated 26 DRB1*04 samples, which had been subtyped using DRB1*04 subtyping kits (Bio-Synthesis), and 26 non-DRB1*04 samples.

2.3.2 Monoclonal Antibodies

Monoclonal antibodies (mAbs) (spent supernatants) were used to detect generic HLA-class I (W6/32, 1:150, ATCC, Manassas, VA, USA) and HLA-DR (L243, 2.4μg/ml, ATCC). Commercially available mAbs were used to detect Ii chain (clone

LN2, 2.5μg/ml, Pharmingen, Mississauga, ON, Canada) and CD3-positive infiltrating T cells (clone UCHT1, 2.5μg/ml, Pharmingen). Negative controls consisted of isotype matched non-specific mouse immunoglobulins (Southern Biotechnology Associates, Inc., Birmingham, AL, USA). Samples that contained HLA-DR+ tumor cells were further evaluated for expression of individual HLA-DRB alleles using a panel of monoclonal antibodies that distinguish HLA-DR polymorphisms (Table 2.1). These included local mAbs NFLD.D1 [232, 233], NFLD.D10 [232], and NFLD.D7 [232], and SFR16 [234] a kind gift from Susan Radka. UK8.1 [235], 7.3.19.1 [236], PL3 [237] and MAD88 were obtained through the 10th International Histocompatibility Workshop (IHW).

To determine optimal antibody concentrations and show that mAbs neither recognized cell type restricted epitopes nor cross-reacted with additional HLA-DRβ allotypes, immunohistochemistry was performed on various cell types with known HLA-DRB allotypes. These included, IFN-γ treated ovarian and breast cancer cell lines, peripheral blood mononuclear cells (from local donors), B cell lines (10th IHW), mouse L-cell fibroblast lines transfected with HLA-DR molecules (11th IHW) and synovial tissue sections (data not shown). All mAbs showed reactivity in concordance with their published specificities. MAD88 was submitted to the 10th IHW as a HLA-DRβ1*08 specific mAb but was found to cross react to alleles of the HLA-DRβ3 (DR52) haplotype. However, additional alleles with which MAD88 reacts were not present in the DRB1*08 tumors in this study.

Table 2.1: HLA-DRβ Allotype Specific mAbs used in this Study

mAb	Isotype	Concentration/ Dilution	HLA-DRβ Specificity
NFLD.D1	IgG1	10μg/ml	β1*04
NFLD.D7	IgG1	1/100	β1*04, 15, 16; β3
NFLD.D10	IgG1	3μg/ml	β1*01, 15, 04[1], 14[1], 09, 10; β5*02
SFR16	IgG2a	1/25	β1*07
UK8.1	IgG2b	1/1000	β1*03, 11, 13, 14[1]
7.3.19.1	IgG2b	1/1000	β1*03, β3
PL3	IgG3	1/400	β1*07, 09; β4
MAD88[2]	IgG	1/100	β1*08, β3

[1]Not all allelic products of this HLA-DR type carry the epitope recognized by this antibody.
[2]This mAb was reported as DRβ1*08 specific in the 10th IHW but shows cross reaction with HLA-DRβ3 alleles.

2.3.3 Immunohistochemistry

Serial frozen sections (8 μm) were fixed in acetone for 10 minutes at –20°C, shipped from the NCIC Manitoba Breast Tumor Bank and stored at –70°C until immunohistochemical staining. After thawing, drying and rehydrating in phosphate buffered saline (PBS) (pH 7.4), sections were treated with 1.5% hydrogen peroxide in PBS for 30 minutes to remove endogenous peroxidases, and nonspecific binding was blocked with 15% goat serum in PBS for 1 hour. Sections were incubated for 1 hour with primary antibody followed by incubation for 30 minutes with goat anti-mouse DAKO EnVision horseradish peroxidase (HRP) labeled polymer (DAKO Diagnostics Canada Inc., Mississauga, ON, Canada). Antibody binding was visualized by incubating with diaminobenzidine + hydrogen peroxide (Sigma, Oakville, ON, Canada) for 5 minutes. The reaction was stopped with water and sections were counterstained in Mayer's haematoxylin. Infiltrating mononuclear cells served as intrinsic positive controls for the immunoreactivity of the monoclonal antibodies.

2.3.4 Immunohistochemistry Interpretation

All slides were coded and independently examined by three readers (J.D.R, S.O., and S.D.). Infiltrating T cells were coded based on estimated numbers: - (no or a few scattered cells); -/+ (small numbers of scattered cells or occasional small aggregates); + (moderate numbers of scattered cells, numerous small aggregates or occasional large aggregates); ++ (large numbers of scattered cells or several large aggregates). For analysis, sections were considered positive for an infiltrating T cell population if scored +

or ++. The distribution of infiltration (within tumor nests and/or throughout the intervening tumor stroma) was also determined.

The percentage of tumor cells expressing HLA-DR, Ii and HLA class I was coded based on comparison to expression levels on inflammatory cells within the same tissue section: - (0-24% tumor cells positive); -/+ (25-49% tumor cells as strong as inflammatory cells or 25-74% tumor cells weaker than inflammatory cells); + (50-100% tumor cells as strong as inflammatory cells or 75-100% tumor cells weaker than inflammatory cells). Since HLA-DR and Ii are not normally expressed on breast epithelial cells, for categorical analysis samples with HLA-DR or Ii up regulated in at least 25% of tumor cells were classified as positive. For HLA class I, only those coded + were considered positive as samples coded – or -/+ were considered to have a substantial down regulation of HLA class I. Likewise, only the percentage of tumor cells strongly positive for HLA class I was used for continuous variable analysis while total percentage of tumor cells positive (weak or strong) was included for HLA-DR and Ii..

2.3.5 Statistical Analysis

Analysis was performed using SPSS Version 10.0 statistical software. Contingency tables were analyzed using Pearson's chi-square analysis or Fisher's exact test for 2x2 tables with expected counts ≤ 5. Non-parametric Mann-Whitney U or Kruskal-Wallis H tests were used when assessing statistical significance of the actual percentages of tumor cells positive for HLA-DR, Ii and HLA class I. All tests were two-sided and differences between groups were considered significant if $p < 0.05$. In the text, mean percentage of positive tumor cells ($\bar{x}_{\%}$) is reported as mean ± standard deviation.

2.4 RESULTS

2.4.1 Tumor cell expression of HLA-DR, Ii and HLA class I molecules is positively associated with T cell infiltration

Breast carcinoma samples were assessed for tumor cell expression of HLA-DR, Ii and HLA class I and estimated numbers of infiltrating T-cells (Table 2.2). Based on categorical values, 20 were classified as HLA-DR+ ($\bar{\times}_{\%}$ = 77% ± 22%), 32 were HLA-DR- ($\bar{\times}_{\%}$ = 3% ± 6%), 28 were Ii+ ($\bar{\times}_{\%}$ = 80% ± 23%), 24 were Ii- ($\bar{\times}_{\%}$ = 5% ± 7%), 36 were HLA class I+ ($\bar{\times}_{\%}$ = 72% ± 31%) and 14 were HLA class I- ($\bar{\times}_{\%}$ = 4% ± 6%).

As shown in Figure 2.1, the mean percent of tumor cells with strong HLA class I expression was significantly decreased in HLA-DR- compared to the HLA-DR+ tumors (Mann-Whitney U test, P=0.003), suggesting a negative effect on the HLA complex on chromosome 6. HLA class I-expression was also decreased in Ii- compared to Ii+ tumors but this difference was not significant. As expected, tumor cell HLA-DR and Ii were co-coordinately regulated in the majority of tumors (Pearson's χ^2=27.857, P<0.0001) with 20 co-expressing HLA-DR and Ii, 24 lacking expression of both and 8 expressing Ii in the absence of HLA-DR. Representative examples of DR+Ii+, DR-Ii-, and DR-Ii+ tumor cells are illustrated in Figures 2.2A, 2.2B and 2.2C, respectively.

Table 2.2: HLA-DRB alleles and immunohistochemistry results for breast carcinoma samples examined in this study.

ID#	HLA-DR Alleles*		Expression by Tumor Cells†			CD3+ infiltrate‡
	DRβ1	Other DRβ	HLA-DR	Ii	HLA class I	
7959	04, 12	β4, β3	+§	-/+§	+§	-/+
9914	0401, 03	β4, β3	+§	+§	-/+	-/+
11023	0401, 03	β4, β3	+§	+§	+§	++§
9822	0401, 09	β4, β4	+§	+§	+§	-/+
8192	0401, 15	β4, β5	+§	-/+§	-	+§
11093	0402, 16	β4, β5	+§	-/+§	+§	-/+
11702	0403, 13	β4, β3	+§	+§	+§	++§
11999	0404, 08	β4	+§	+§	+§	++§
10881	0404, 13	β4, β3	+§	+§	+§	++§
8043	0407, 11	β4, β3	+§	+§	+§	+§
11214	0407, 15	β4, β5	+§	+§	+§	++§
11710	0401, 0404	β4, β4	-/+§	+§	+§	-/+
11838	0401, 08	β4	-/+§	+§	+§	-/+
11696	04, 04	β4, β4	-	-	-	-/+
11243	0401, 0404	β4, β4	-	-	+§	+§
9112	0401, 03	β4, β3	-	-	-	-
8183	0401, 13	β4, β3	-	-	+§	-/+
11115	0401, 15	β4, β5	-	+§	-/+	-
12023	0401, 15	β4, β5	-	-	+§	-/+
7952	0402, 01	β4	-	+§	+§	-/+
8824	0404, 13	β4, β3	-	-	+§	+§
11246	0405, 01	β4	-	-	+§	++§
10960	0408, 0408	β4, β4	-	-	-/+	-/+
11770	0408, 0408	β4, β4	-	-	+§	+§
7963	0408, 11	β4, β3	-	-/+§	-/+	-/+
10871	0423, 07	β4, β4	-	-	NR	-
11644	01, 10	-	+§	+§	+§	+§
8196	03, 03	β3, β3	+§	+§	+§	++§
10970	07, 08	β4	+§	+§	+§	++§
11353	08, 16	β5	+§	+§	+§	++§

ID#	HLA-DR Alleles*		Expression by Tumor Cells†			CD3+ infiltrate‡
	DRβ1	Other DRβ	HLA-DR	Ii	HLA class I	
10842	09, 11	β4, β3	+§	+§	+§	+§
11393	13, 14	β3, β3	+§	+§	+§	-/+
11603	13, 15	β3, β5	+§	+§	+§	+§
9930	01, 15	β5	-	-/+§	-/+	-/+
11740	03, 13	β3, β3	-	-	-	-
7953	03, 07	β3, β4	-	-	-	-/+
8795	07, 07	β4, β4	-	-	NR	++§
9110	07, 13	β4, β3	-	-	+§	-/+
10666	07, 07	β4, β4	-	+§	+§	++§
11000	07, 10	β4	-	-	+§	-
11148	07, 11	β4, β3	-	-	+§	-/+
11288	07, 11	β4, β3	-	-	+§	-
11372	07, 08	β4	-	-	+§	+§
12020	07, 16	β4, β5	-	-	-	+§
11982	09, 15	β4, β5	-	-/+§	+§	++§
8740	11, 16	β3, β5	-	-/+§	-/+	+§
11778	11, 14	β3, β3	-	-	+§	-/+
10528	12, 13	β3, β3	-	+§	-/+	-/+
11692	13, 13	β3, β3	-	-	+§	+§
8772	15, 16	β5, β5	-	-	+§	+§
11084	15, 15	β5, β5	-	-	-	+§
11802	15, 16	β5, β5	-	-	+§	+§
No. Samples (%)		-	32 (61.5)	24 (46)	14 (28)	25 (48)
		+	20 (38.5)	28 (54)	36 (72)	27 (52)
		Total	52	52	50	52

* Since samples were DNA typed for HLA-DR by PCR-SSP using HLA class II typing kits, samples that typed for only one HLA-DRβ1 allele were considered homozygous.

† Tumor cell expression was classified as - (<25% positive); -/+ (25-74% weak or 25-49% strong); or + (75-100% weak or 50-100% strong) as defined in text. Samples considered positive by IHC are shaded in gray; nr = no result (due to loss of tissue section during staining procedure).

‡ CD3+ infiltrating cells were classified as - (no or very few); -/+ (small numbers); + (moderate numbers); or ++ (large numbers) as defined in text.

§ Samples categorically classified as positive by IHC, as defined in methods.

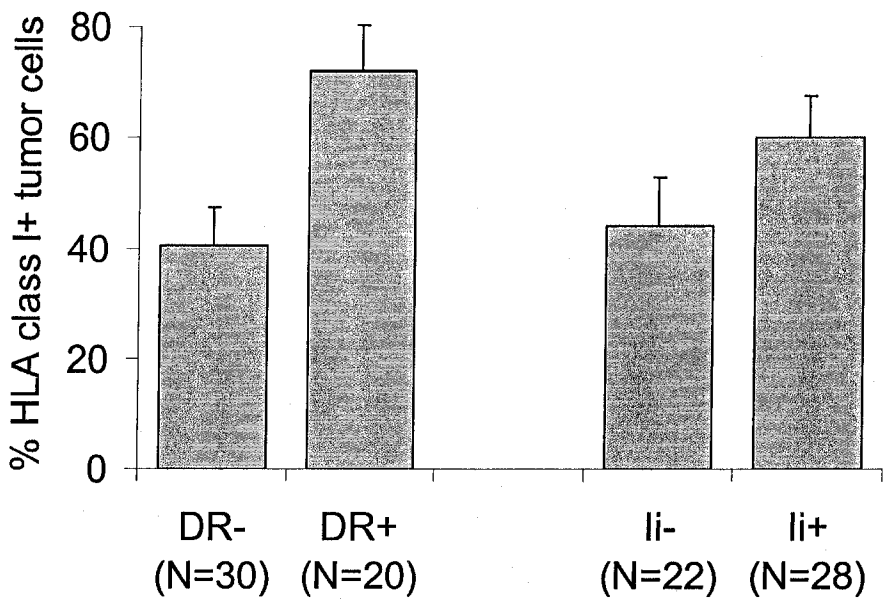
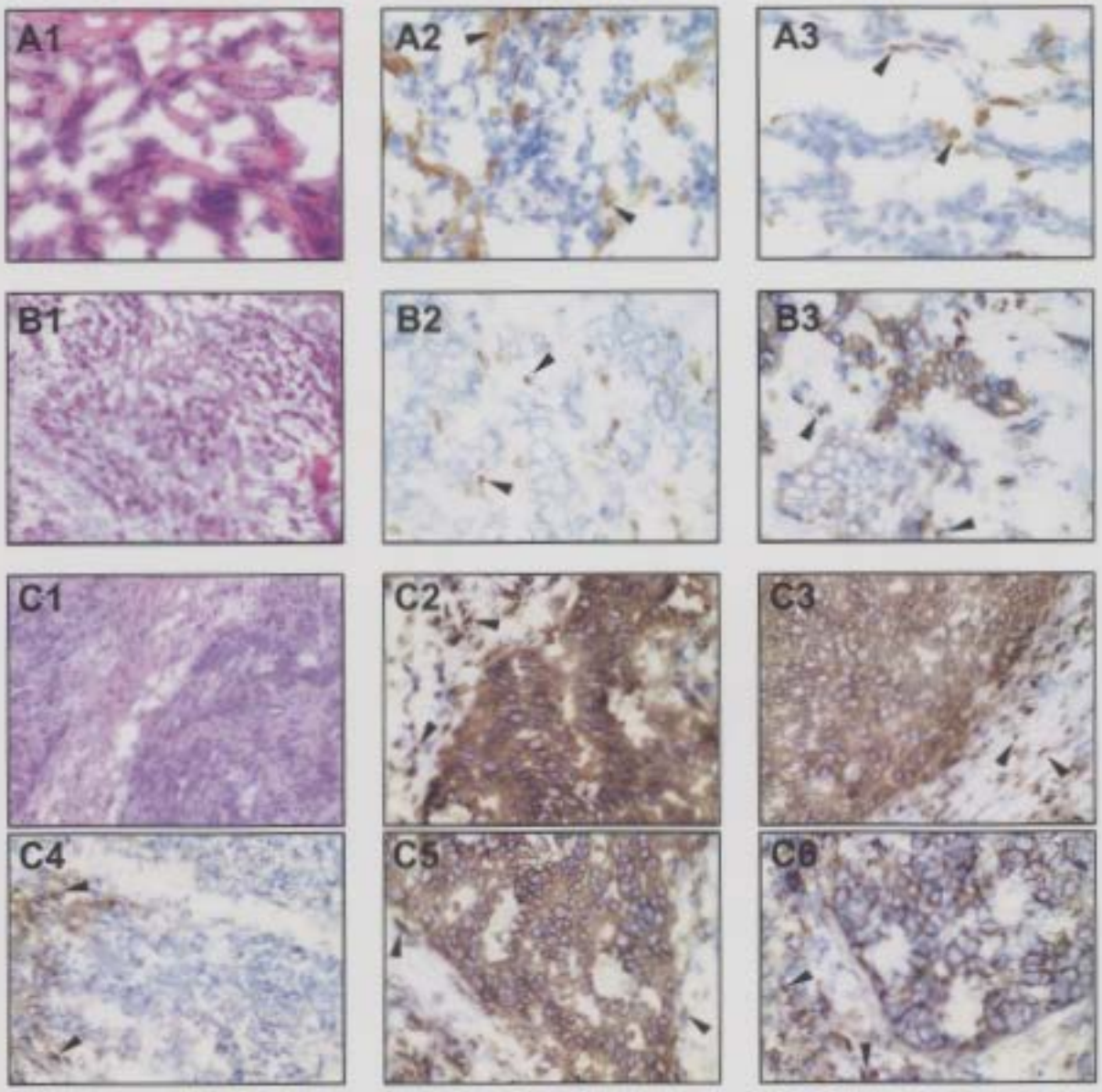


Figure 2.1: HLA class I expression is decreased in both HLA-DR- (Mann-Whitney U test P=0.003) and Ii- (Mann-Whitney U test, P=0.069) tumors. Data shown are the mean % of tumor cells with HLA class I expression as strong as inflammatory cells ± SEM.

Figure 2.2: HLA-DR and Ii expression by tumor cells in breast carcinoma samples detected by indirect immunohistochemistry showed different expression patterns. **A:** The most common phenotype was tumor cells negative for HLA-DR (A2) and Ii (A3). **B:** A minor subset of HLA-DR negative samples (B2) did express Ii (B3). C: Samples with HLA-DR expressing tumor cells (C2) also expressed Ii (C3). Further analysis of samples with HLA-DR positive tumor cells showed that carcinoma cells discordantly express HLA-DRB alleles. In this representative example, tumor cells did not express the DRβ1*08 allotype (C4) although tumor cells showed homogeneous expression of the DRβ1*04 (C5) and DRβ4 (C6) allotypes. Hematoxylin and eosin stained sections from each sample are depicted (A1, B1 and C1). Infiltrating inflammatory cells and stromal cells served as intrinsic positive controls for antibody binding (arrowheads). Original magnification, X100 (B1, C1), X200 (A1) and X400 for remaining photos.

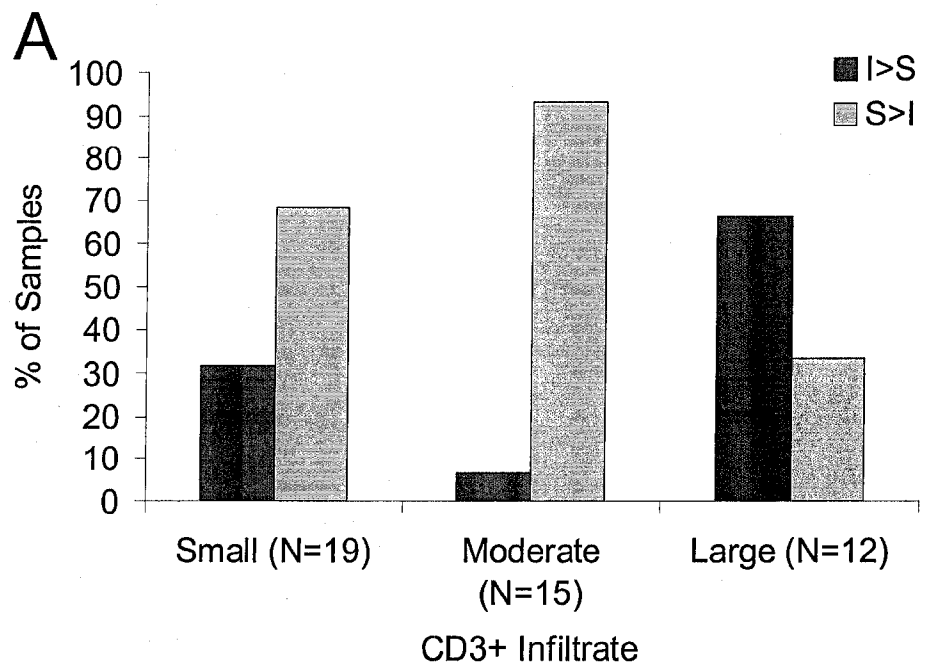

A1
A2
A3
B1
B2
B3
C1
C2
C3
C4
C5
C6

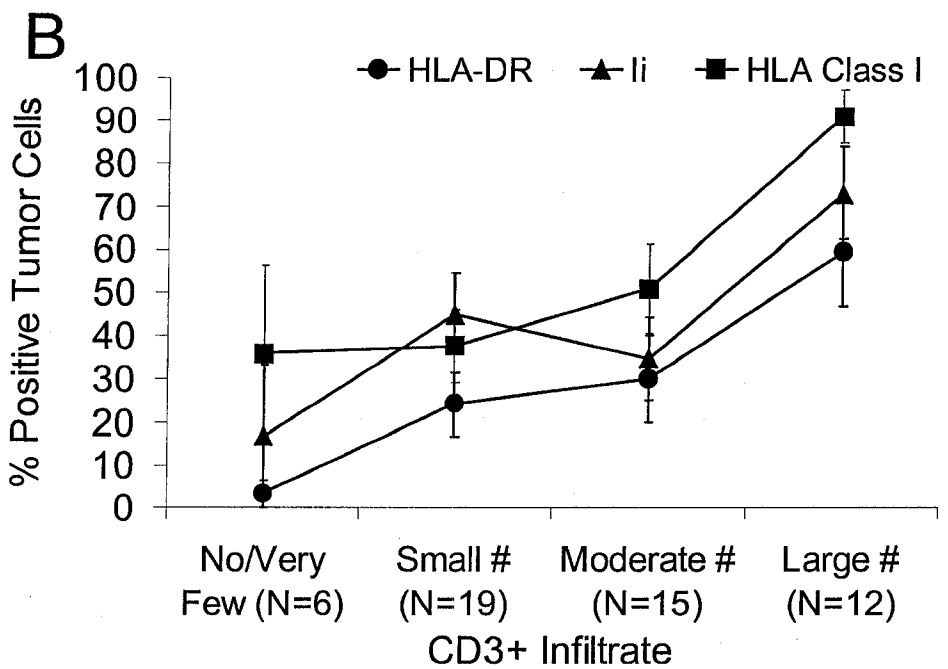
Infiltrating CD3+ T cells were detected, both intratumorally and throughout the intervening tumor stroma, in 46 tumors of which 27 contained a moderate to large T cell infiltrate (Table 2.2). The location of infiltrating T cells was predominately stromal in those tumors with small to moderate CD3+ infiltrates but intratumoral for the majority of tumors with large CD3+ infiltrates (Figure 2.3A). Of the 46 tumors with infiltrating T cells, those with intratumoral T cells exceeding stromal T cells had a higher mean percentage of HLA-DR+ tumor cells ($\bar{\times}_{\%}$ = 48%±43 versus 25±37%; Mann-Whitney U test, P=0.161, not depicted) and intratumoral T cells were detected in all HLA-DR+ tumors (Fisher's exact test P=0.017). Overall, as shown in Figure 2.3B, CD3+ T cell infiltration was significantly associated with tumor cell expression of HLA-DR, Ii and HLA class I, independent of location.

2.4.2 HLA-DRB alleles influence tumor cell expression of HLA-DR and Ii and infiltrating T cells

To determine whether DRB1*04 and/or individual DRB1*04 alleles influenced tumor cell expression of HLA-DR and Ii, these factors were compared between the DRB1*04 and non-DRB1*04 tumors. As shown in Figure 2.4, 13/26 DRB1*04 tumors were HLA-DR+, 16/26 were Ii+ and 11/26 contained moderate to large T cell infiltrates. This trend was reversed in non-DRB1*04 tumors as only 7/26 were HLA-DR+, 12/26 were Ii+, but a greater number 16/26 contained a substantial T cell infiltrate. However, both DRB1*04 and non-DRB1*04 tumors contained a higher percentage of HLA-DR+ tumor cells when intratumoral T cells exceeded stromal T cells (not depicted).

Figure 2.3: Infiltrating CD3+ T cells are contained within the tumor stroma and intratumorally and associate with tumor cell expression of HLA-DR, Ii and HLA class I. **A)** Location of infiltrating CD3+ T cells in breast carcinoma samples. Tumors with large T cell infiltrates contain predominantly intratumoral T cells (Pearson's χ^2=10.937; P=0.004); I = intratumoral, S = stromal. **B)** Increased CD3+ T cell infiltrate was significantly associated with an increased percentage of tumor cells expressing HLA-DR (Kruskal-Wallis χ^2=12.0, P=0.007), Ii (Kruskal-Wallis χ^2=10.9, P=0.012) and HLA class I (Kruskal-Wallis χ^2=17.0, P=0.001). Data are shown ± the standard error of the mean.


A
100
90
80
70
60
50
40
30
20
10
0
% of Samples
I>S
S>I
Small (N=19)
Moderate (N=15)
Large (N=12)
CD3+ Infiltrate


B
100
90
80
70
60
50
40
30
20
10
0
% Positive Tumor Cells
HLA-DR
Ii
HLA Class I
No/Very Few (N=6)
Small # (N=19)
Moderate # (N=15)
Large # (N=12)
CD3+ Infiltrate

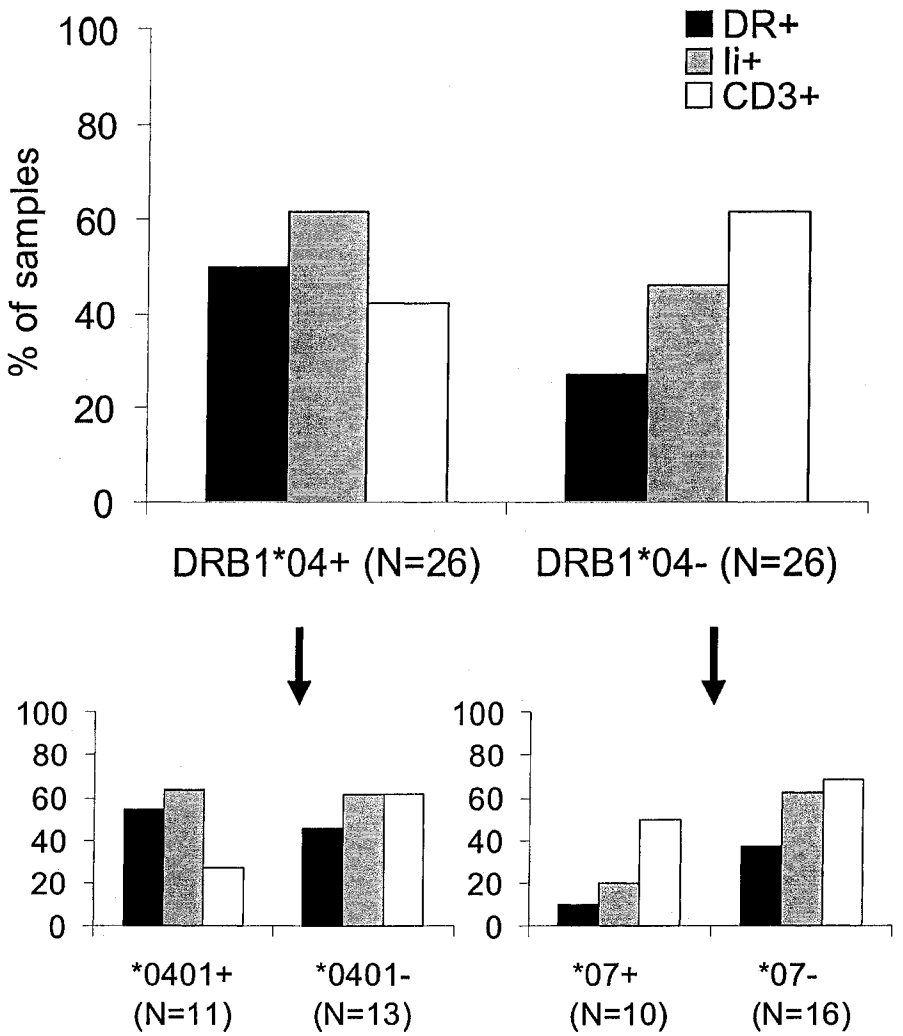


Figure 2.4: HLA-DR allotype influences tumor cell expression of HLA-DR. Indirect immunohistochemistry was used to detect tumor cell expression of HLA-DR, and Ii and T cell infiltration in breast carcinomas. HLA-DR and Ii expression was increased in DRB1*04 tumors (HLA-DR: Pearson's χ^2=2.925; *P*=0.087; Ii: Pearson's χ^2=1.238; *P*=0.266). Decreased T cell infiltration in DRB1*04 tumors, was attributable to the DRB1*0401 tumors (Pearson's χ^2=2.818, *P*=0.093). Decreased HLA-DR and Ii in non-DRB1*04 tumors was largely influenced by the DRB1*07 allotype (Fisher's exact test *P*=0.190 for HLA-DR and *P*=0.051 for Ii).

Furthermore, while more DRB1*04 tumors were HLA-DR+ based on categorical data, within the HLA-DR+ tumors, the mean percentage of tumor cells expressing HLA-DR was lower in DRB1*04 compared to non-DRB1*04 tumors ($\bar{x}_{\%}$ = 68±22% versus 91±11%, Mann-Whitney U test, P=0.030; not depicted), possibly reflecting the decreased numbers of infiltrating T cells.

DRB1*04 samples were further analyzed for potential differences in HLA-DR-expression and T-cell infiltration in tumors carrying the most common allele, DRB1*0401, present in 11/24 samples that were subtyped. Surprisingly, only 3/11 contained moderate to large T cell infiltrates compared to 8/13 of the remaining DRB1*04 tumors (Figure 2.4 inset). However, decreased T-cell infiltration in DRB1*0401 tumors was significant only if compared to that in non-B1*0401 tumors in the total set (Pearson's χ^2=4.056, P=0.044). Although categorical expression of HLA-DR and Ii by DRβ1*0401 tumors was similar to that observed for all DRB1*04 tumors (Figure 2.4), the mean percentage of HLA-DR+ tumor cells was decreased compared to other DRB1*04 tumors ($\bar{x}_{\%}$ = 63±24% versus 77±21). Again, this likely reflects decreased numbers of infiltrating T cells.

Within the non-DRB1*04 subset, we made the unexpected observation that only 1/10 DRB1*07 samples was HLA-DR+ and 2/10 were Ii+ compared to 6/16 and 9/16, respectively, of non-DRB1*07 tumors (Figure 2.4 inset). Furthermore, the only DRB1*07 tumor within the DRB1*04 subset was also DR-Ii-. This poor expression of HLA-DR and Ii by DRB1*07 tumors was statistically significant when compared to non-DRB1*07 tumors in the total sample (Fisher's exact two-sided test P=0.035 (HLA-DR); P=0.015 (Ii)). T-cell infiltration was somewhat decreased in DRB1*07 tumors, but this

was not significant when compared to non-DRB1*07 tumors within the non-DRB1*04 subset or within the total sample.

Examination of the DR51, DR52 and DR53 haplotype groups revealed no significant associations with HLA expression by tumor cells or T cell infiltration (not depicted), suggesting that differences in HLA-DR expression were influenced by individual HLA-DRB1 alleles and not by groupings of haplotypes based on additional shared HLA-DRB alleles.

2.4.3 HLA-DR+ tumor cells discordantly express HLA-DRB allelic products

Additional tissue was available to evaluate 15 of the HLA-DR+ samples for expression of individual HLA-DRB alleles (Table 2.3). At least 6/15 tumors did not express all HLA-DRB alleles. A representative example of the discordant expression of HLA-DR allotypes is given in Figure 2.2C depicting sample 11999 which contained tumor cells negative for DRβ1*08 (Figure 2.2C4) while 100% of tumor cells expressed the DRβ1*04 (Figure 2.2C5) and DRβ4 (Figure 2.2C6) alleles. Furthermore, in each tumor section analyzed, the relevant mAbs bound to infiltrating inflammatory cells (Figure 2.2C, arrows) suggesting that the relevant allele was not up regulated on the tumor cells. These 15 HLA-DR+ samples carried a total of 57 HLA-DRB alleles, of which expression could be determined for 43 (9 DRβ1*04, 15 other DRβ1, 9 DRβ3 (DR52), 7 DRβ4 (DR53) and 2 DRβ5 (DR51) alleles). Interestingly, the DRβ1*04 allotype was expressed in all nine DRB1*04 samples compared to 11/15 non-DRβ1*04

Table 2.3: HLA-DRβ Alleles Detected on HLA-DR+ Tumor Cells

Sample ID	HLA-DRB alleles Expressed by HLA-DR+ Tumor Cells*		
	Detected	Undetected	Undetermined
11702	β1*0403, β1*13, DR52, DR53	0	0
8043	β1*0407, β1*11, DR52, DR53	0	0
8196	β1*03, DR52	0	β1*03, DR52
10881	β1*0404, β1*13, DR52, DR53	0	0
11353	β1*08, β1*16, DR51	0	0
9822	β1*0401	0	β1*09, DR53, DR53†
11393	β1*14, DR52	0	β1*13, DR52
11023	β1*0401, β1*03, DR53	0	DR52
11603	β1*13, β1*15, DR52	0	DR51
9914	β1*0401, DR52	β1*03, DR53	0
10970	β1*07	β1*08	DR53‡
11999	β1*0404, DR53	β1*08	0
7959	β1*04	DR52	β1*12‡, DR53§
11093	β1*0402	DR51, DR53	β1*16
10842	-	β1*11, DR52	β1*09; DR53†

* Allele expression was classified as described in text: positive (≥ 25% tumor cells positive); negative (0-24% tumor cells positive) or undetermined (expression could not be determined using available panel of antibodies). DR51 = DRβ5; DR52 = DRβ3; DR53 = DRβ4.

For samples that are homozygous for a particular allotypes, expression of only one of the alleles can be ascertained using the allotype specific mAbs.

† Tumor cells stained positively with the PL3 antibody, however, this antibody cannot distinguish between the DRβ1*09 and DRβ4 allelic products.

‡ Allele is likely expressed due to differences in % of tumor cells positive for other alleles and % positive for total HLA-DR.

§ Tumor cell expression could not be accurately assessed as DR53 was not expressed by infiltrating inflammatory cells in this tumor (possible null allele).

alleles. The DRβ3, DRβ4 and DRβ5 allotypes were expressed in 7/9, 5/7 and 1/2 tumors, respectively. Discordant allelic expression was unique to the individual tumor as each of the allotypes not expressed was detected within another tumor. Importantly, the HLA-DR+ sample 10970 did express its DRβ1*07 allele, demonstrating that this allotype can be up regulated by tumor cells in breast carcinoma.

2.5 DISCUSSION

A major goal of this study was to determine if tumor cell expression of HLA-DR and the presence of infiltrating T-cells in breast carcinoma are influenced by an individual's HLA-DRB alleles. Secondly, since most individuals are heterozygous for DRB1 and associated structural DRB genes, we postulated selective expression of these alleles in the subset of tumors that were HLA-DR+. Using an equal number of randomly selected DRB1*04 and non-DRB1*04 samples and a panel of mAbs that distinguished polymorphic epitopes on HLA-DR allotypes, we made three novel findings: a) DRB1*04 tumors are more frequently HLA-DR+ but contain smaller numbers of infiltrating T-cells than non-DRB1*04 samples; b) within the non-DRB1*04 tumors, only 9% of tumors that carried DRB1*07 express DR; c) at least 40% of tumors that were HLA-DR+ fail to express one or more of their HLA-DR allotypes.

Since tumor cell expression of HLA-DR positively associated with infiltrating T-cells in the whole sample, our finding that DRB1*04 tumors, especially those carrying the DRB1*0401 allele, were more frequently HLA-DR+ despite having reduced T-cell infiltration was unexpected. However, all DRB1*0401 HLA-DR+ tumors contained

some intratumoral T cells, whose presence may explain why categorical numbers of HLA-DR+ tumors were not decreased. On the other hand, reduced numbers of T-cells were reflected by the decreased mean percentage of HLA-DR+ tumor cells in DRB1*0401 tumors. Thus, it seems that up-regulation of HLA-DR on tumor cells is related not only to numbers of infiltrating T cells but also to their location within the tumor. This may have important implications for disease outcome in breast carcinoma as the presence of intratumoral T cells independently predicted for improved disease-free and overall survival in late stage ovarian cancer patients [193].

The most novel finding from this study was the absence of HLA-DR-expression by 10/11 DRB1*07 tumors, despite possessing DRB alleles that were expressed in non-DRB1*07 tumors. Since HLA-DR was strongly expressed on stromal and inflammatory cells in all samples, we are confident that this phenomenon is not a technical artifact. An attractive explanation is loss of chromosome 6p, the most frequent mechanism contributing to HLA haplotype loss [238] combined with mutations/deletions of the remaining HLA genes resulting in the loss both haplotypes. However, we favor an, as yet, undefined epigenetic effect for two reasons: i) HLA class I was strongly expressed in at least 6/10 DRB1*07 HLA-DR- tumors suggesting that in those tumors both HLA haplotypes were not lost; ii) Ii, which is not located on chromosome 6, and which is coordinately regulated with HLA class II genes, was scarcely expressed in DRB1*07 tumor cells. Defects in one or more factors, such as the class II transactivator (CIITA), required for transcription of HLA class II and Ii, have been reported in various carcinoma cell lines that do not up regulate HLA class II expression [239, 240]. It would be surprising, however, if defective CIITA was more frequent in DRB1*07 tumors, but the

effect could be indirect since CIITA activation in tumor cells requires IFN-γ whose effects are known to be inhibited by TGF-β [241]. Thus, if DRB1*07 tumors preferentially stimulate immunosuppressive T-cells, it could explain poor tumor cell expression of all HLA-DR allotypes as well as Ii, despite the presence of infiltrating T-cells. While we have not addressed the phenotype of the infiltrating T cells in this study, others have found that breast and pancreatic carcinoma patients have increased numbers of CD4+CD25+ regulatory T cells, which potentially serve as another mechanism for tumor immune escape [184].

Mechanisms responsible for discordant HLA-DR allelic expression in HLA-DR+ tumor cells are likely to be different from those responsible for complete absence of HLA-DR-expression. In the 15 HLA-DR+ tumors analyzed, all DRB1*04 alleles were expressed, but other alleles, whether present in DRB1*04 or non-DRB1*04 tumors were inconsistently expressed. In fact, the one DRB1*07 sample expressed its DRB1*07 allele but not its DRB1*08 allele, indicating that DRB1*07 can be up regulated on breast tumor cells. Loss of heterozygosity (LOH) at chromosome 6p21.3 is a common mechanism leading to HLA class I haplotype loss in several human cancers, including laryngeal [242] and colorectal carcinomas [243] and may represent one mechanism responsible for discordant allelic HLA-DR-expression in the tumors in which at least one HLA-DR allele was not expressed. For example, LOH may be responsible for the loss of the DRB1*08 haplotype in samples 11970 and 11999. However, the pattern of allelic expression for sample 9914 suggests an alternative mechanism as one allele from each inferred haplotype is expressed. Differential expression of HLA-DR by tumor cells may also be modulated by polymorphisms in the X and Y-boxes of HLA-DRB promoters [244, 245].

Comparing HLA-DRB promoter polymorphism related differences in transcriptional activity to levels of HLA-DRB mRNA suggest that differential HLA-DRB gene expression is regulated at both transcriptional and post-transcriptional levels [118, 119]. Thus, factors produced during tumorigenesis may contribute to HLA-DRB gene regulation.

The implications of discordant expression of Ii and HLA-DR, in our and other studies of breast carcinoma [146, 151] remain unclear. Independent evaluation of Ii expression by tumor cells has revealed an association with a poor prognosis in colon carcinoma [160] and gastric carcinoma [161]. Others have reported that lack of HLA class II and Ii expression correlated with down regulation of tumor cell HLA class I [146], a finding supported in this study. Indeed, the degree of HLA class I loss is likely underestimated as most tumors classified as HLA class I+ using the W6/32 mAb do indeed contain altered HLA class I phenotypes [225].

The small sample size and biased selection for DRB1*04 in this study precludes a meaningful statistical analysis of tumor cell expression of HLA-DR and Ii and T-cell infiltration with prognostic indicators. However, there were some notable trends such as reduced ER levels in non-DRB1*04 tumors that expressed HLA-DR and Ii compared to ER levels in DRB1*04 DR+ tumors, suggesting that ER may be implicated in regulating HLA-DR expression in these tumors. Furthermore there was a trend for lower tumor grade in those tumors that expressed HLA-DR in the absence of a substantial T cell infiltration, suggesting HLA-DR expression in the absence of T cell-derived cytokines may result from molecular changes during malignant transformation. Overall, in concordance with earlier studies [162, 177], HLA-DR was significantly associated with T

cell infiltration, supporting the hypothesis that HLA-DR is induced on tumor cells in response to the cytokine milieu of the tumor microenvironment.

In conclusion, expression of HLA-DR by breast carcinoma cells appears to be a complex phenomenon mediated by many interacting factors within and outside the tumor. These include polymorphisms within the non-coding and coding regions of HLA-DRB alleles that directly influence anti-tumor T cell responses and production of cytokines known to modulate tumor cell expression of HLA-DR. Other variables, likely to affect HLA-DR expression, include the differentiation state of the tumor, hormones and hormone receptors. Discordant expression of particular HLA-DRB alleles in HLA-DR+ tumors and the association of DRB1*07 with HLA-DR- tumors illustrate the importance of examining specific expression of HLA-DRB alleles in future studies aimed at elucidating the biological and prognostic significance of HLA-DR expression in carcinoma.

CHAPTER 3: TUMOR CELL EXPRESSION OF HLA-DRB1 ALLOTYPES HAS IMPORTANT IMPLICATIONS FOR ANTI-TUMOR IMMUNITY AND SURVIVAL IN INVASIVE BREAST CARCINOMA PATIENTS†

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3.1 ABSTRACT

There is strong experimental evidence to support a role for HLA-DR expression by tumor cells in the induction of an anti-tumor T cell response. However, studies attempting to relate the two have yielded conflicting results. To determine if allelic differences in HLA-DR antigens influence anti-tumor immunity and resultant patient outcome, 121 breast carcinoma lesions with prognostic information were DNA-typed for HLA-DRB alleles. Tumor cell HLA-DR expression and CD4+ tumor infiltrating lymphocytes (TIL) were examined using indirect immunohistochemistry. HLA-DR+HLA-DRB1*04+ tumors had decreased tumor size and independently predicted improved distant recurrence-free survival (dRFS) and disease-specific survival (DSS) while HLA-DRB3 and HLA-DRB1*13 tumors that express HLA-DR were larger, more frequently had lymph node metastasis and independently associated with decreased dRFS. Variability in intratumoral cytokine levels suggest HLA-DRB1*04 expression may promote a favorable anti-tumor T_H1-type response marked by IFN-γ production while expression of HLA-DRB3 and HLA-DRB1*13 may promote an immunosuppressive immune response, marked by elevated TGF-β1, allowing tumor outgrowth. Immunohistochemical evaluation using HLA-DRβ allotype-discriminating antibodies confirmed our previous finding that the majority of HLA-DR+ tumors exhibit differential DRβ allotypic expression, with HLA-DRβ1*04 preferentially expressed. In non-DRB1*04 tumors, differential DRβ allotype expression associated with decreased dRFS and DSS, suggesting lack of expression of particular DRβ allotypes may represent an immune evasion strategy employed by tumor cells, similar to that observed with HLA class I antigens. Ongoing studies aimed at elucidating the mechanisms that control HLA

class II expression in breast carcinoma will provide important insights into host-tumor interactions and immune evasion strategies employed by tumor cells.

3.2 INTRODUCTION

Immune-mediated eradication of solid tumors involves CD4+ tumor infiltrating lymphocytes (TIL) that recognize tumor peptides, presented in the context of HLA class II antigens (HLA-DR, -DP, -DQ) [166, 167]. Extensive polymorphism of HLA-class II molecules, localized to the peptide binding sites, determines peptide selection and CD4-recognition while limited polymorphism in class II promoter regions influences expression levels on various cell types [118, 119]. Class II molecules are abundantly expressed on conventional antigen-presenting cells (APC) such as dendritic cells, activated macrophages and B-cells, but may be up regulated on various cell types, including breast cancer cells, in response to immunomodulating agents and cytokines [134-136]. Thus, the efficacy of a CD4+ T cell specific anti-tumor response is not only influenced by allelic differences in antigen-presenting molecules but also by the cells on which the HLA class II/peptide complexes are presented.

Breast carcinomas are frequently infiltrated by HLA-DR expressing inflammatory cells and CD4+ TIL [186]. Furthermore, a subset of breast carcinomas express HLA-DR antigens on their tumor cells and accumulating experimental evidence supports a role for HLA class II expression by tumor cells in the induction of an anti-tumor T cell response [89, 138, 168, 170]. CD4+ TIL isolated from breast cancer patients have been shown to secrete T_H1-type cytokines in response to autologous tumor cells [170] and both T_H1 and T_H2-type cytokine producing TIL are detected in breast carcinomas [191]. In one study,

up to 20% of CD4+ TIL expressed an immunoregulatory (T_{reg}) phenotype, and secreted TGF-β1 and IL-10 in response to non-specific stimulation and were capable of *in vitro* suppression of activated CD4+CD25- and CD8+CD25- T cells [184]. Since allelic differences in HLA-DR antigens influence CD4+ T cell activation and ensuing cytokine production [124, 246], in the context of carcinoma, the effectiveness of the anti-tumor immune response is surely dependent on the HLA-DRβ allotypes expressed on tumor cells and/or infiltrating APC.

Although several studies have shown that HLA-DR molecules are preferentially expressed on HLA class II-positive breast carcinomas, there is no clear consensus on the prognostic value of HLA-DR expression by breast tumor cells [146-148, 152, 155, 156, 159, 247]. Discrepancies regarding associations of prognostic factors and survival with HLA-DR+ tumor cells may be methodological in that previous studies examined generic HLA-DR expression in the absence of information on carriage and expression of the patient's HLA-DRB alleles. Such studies are complicated by a large number of HLA-DRB1 alleles, which are further organized into haplotype groups based on their linkage with additional structural HLA-DRB genes and HLA-DRB pseudogenes. HLA-DRB1*15 and 16 alleles are expressed in association with HLA-DRB5 alleles (DR51); HLA-DRB1*03, 11, 12, 13 and 14 with HLA-DRB3 alleles (DR52); HLA-DRB1*04, 07 and 09 with HLA-DRB4 (DR53); while HLA-DRB1*01, 10 and 08 are not in association with any other expressed HLA-DRB genes [70]. Since HLA antigens are co-dominantly expressed an individual can express up to four different DRβ allotypes [70], and expression of only one of these will yield a positive result when tumor cells are analyzed for generic DR-expression.

We previously reported that most HLA-DR+ breast carcinoma cells differentially express their HLA-DRβ allotypes with DRB1*04 preferentially expressed [248]. Furthermore, we have recently identified that carriage of HLA-DRB allotypes belonging to the HLA-DR52 (DRB3) haplotype group, in particular HLA-DRB1*13, associates with decreased survival in breast carcinoma (Drover et al., manuscript in preparation). This implies a role for HLA-DRβ allotypes in tumor immune responses, and consequently outcome, in patients with breast carcinoma. To determine the implications of these findings, we investigated whether HLA-DRB allotypic differences influence tumor cell HLA-DR expression, immune responsiveness in terms of CD4+ T cell infiltration and cytokine production and levels of the immunoregulatory T cell marker FOX-P3, or have any implications for prognosis and outcome.

3.3 MATERIAL AND METHODS

3.3.1 Study subjects

One-hundred and four primary and 17 recurrent breast tumor lesions were randomly selected, based on tissue availability, from a larger sample with prognostic and outcome information, obtained from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada), with approval of the local Human Investigation Committees. Tissue samples were subjected to immunohistochemical evaluation and following exclusion of samples where tumor tissue comprised <10% of the section area, immunohistochemical information was available for 99 primary and 15 recurrent lesions. As several biological changes mark progression to metastatic disease, associations of immunological parameters with prognosis and survival were assessed for primary lesions only. To verify

that tumor samples selected for immunohistochemical analysis were representative of the entire set of primary tumors, for which prognostic information was available, associations of HLA-DRB alleles with prognostic parameters were assessed in both tumor sets (Appendix I).

Primary treatment information was available for 97 patients, of which 21 were treated with surgery only and the remainder received adjuvant therapy. Information on tumor type was available for 96 samples of which 81 were infiltrating ductal (IDC), 13 infiltrating lobular (ILC) and 2 mixed IDC+ILC. Tumor grade was available for 91 tumors of which 12 were Grade I, 44 were Grade II and 35 were Grade III, determined using the Nottingham grading system [6]. Tumor size was available for 96 patients (mean±SD = 3.3±2.3), and was categorized as small (≤2 cm, n=24) or large (>2 cm, n=72). Lymph node (LN) status was available for 98 patients and obtained using pathological determination for LN+ tumors and pathological and/or clinical determination for LN- tumors. Fifty-three patients were LN+ (1-3 nodes positive, N=38; >3 nodes positive, N=15). Ninety-five tumors were staged, according to AJCC guidelines [8], and there were 12 Stage I, 63 Stage II, 17 Stage III and 3 Stage IV tumors. The age at diagnosis ranged from 32 to 86 years (mean±SD = 58.8±14.4 years; median 60 years). Estrogen and progesterone receptor levels were determined by ligand binding assay and values ranged from 0 to 331 fmol/mg (mean±SD = 40.0±60.8 fmol/mg; median 13.1 fmol/mg) and 0 to 1088 fmol/mg (mean±SD = 59.6±142.4; median 15.8 fmol/mg), respectively. Tumors were stratified based on a cutoff of 10 fmol/mg and there were 43 tumors with ER<10 fmol/mg and 56 with ER ≥ 10 fmol/mg and 34 with PR<10 fmol/mg and 65 with PR ≥ 10 fmol/mg. Her-2/neu expression was assessed in 89 tumors, as described below, and 19 (21.3%) tumors over-expressed Her-2/neu (code 3+). Survival

data was available for all patients with a median follow-up time of 60 months (mean±SD = 57.8±30.3; range 2-127 months). During this follow-up period, 34 patients suffered a distant relapse (mean time to relapse±SD = 23.7±17.4 months; range 0-64 months) and 30 patients died from breast cancer (mean time to death±SD = 31.2±20.6 months; range 2-90 months). The prognostic significance of clinicopathological parameters is provided in Appendix II.

3.3.2 DNA extraction and HLA class II typing by PCR-SSP

DNA was isolated from thawed, fresh-frozen breast tumor tissues by homogenization in DNAzol (Gibco BRL, Rockville, MD). DNA was typed for generic HLA-DRB and HLA-DRB1 and HLA-DRB3 alleles by polymerase chain reaction with sequence-specific primers (PCR-SSP) using Micro SSP^{TM} (One Lambda, Inc., Canoga Park, CA, USA) or Fastype^{TM} System (Bio-Synthesis, Inc., Lewisville, TX, USA) typing kits.

3.3.3 Immunohistochemistry

Serial frozen sections (8 μm) were fixed in acetone for 10 minutes at –20°C, shipped from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada) and stored at –70°C until immunohistochemical staining. Hematoxylin and eosin staining was performed on one section for each breast carcinoma and samples where tumor cells comprised <10% of the section area were excluded from analysis. Indirect immunohistochemistry was performed and interpreted as previously described (Chapter 2) [248]. In brief, tumors with at least 25% of tumor cells expressing HLA-DR were classified as HLA-DR+ and the percentage of tumor cells expressing HLA class I was

assessed as an indirect measure of integrity of HLA loci. Tumor cells were identified using anti-cytokeratin mAb (AE1/AE3, 5μg/ml, DakoCytomation, Mississauga, ON, Canada). Monoclonal antibodies in the form of spent supernatants were used to detect generic HLA-class I (W6/32, 1:150, ATCC, Manassas, VA, USA) and HLA-DR (L243, 2.4μg/ml, ATCC). CD4+ TIL were detected using anti-CD4 mAb (clone RPA-T4, 0.6μg/ml, Pharmingen) and semi quantitatively scored as none (-), small (-/+), moderate (+) or large numbers (++), based on numbers of scattered cells and/or size of focal aggregates, as previously described (Chapter 2) [248]. For analysis, those coded – or -/+ were classified as $CD4+^{Lo}$ and those coded + or ++ were classified as $CD4+^{Hi}$. Negative controls consisted of isotype matched non-specific mouse immunoglobulins (Southern Biotechnology Associates, Inc., Birmingham, AL, USA). Tumor cell expression of Her-2/neu was determined with specific mAb (clone CB11, 1/100; clone N12, 2 μg/ml, Neomarkers, Quebec, Canada) in 89 primary tumors and coded as 0 (<10% with membrane staining), 1+ (≥10% with weak, incomplete membrane staining), 2+ (≥10% with weak to moderate complete membrane staining) or 3+ (≥10% with strong complete membrane staining). As some tumors coded 2+ by IHC will not show gene amplification by fluorescent in situ hybridization [249], only those tumors coded 3+ were considered clinically positive for analysis.

Expression of individual HLA-DRβ allotypes was determined using a panel of monoclonal antibodies that distinguish HLA-DR polymorphisms (Supplementary Table 3S1). These included local mAbs NFLD.D1 [232, 233], NFLD.D10 [232], and NFLD.D7 [232], and SFR16 [234], a kind gift from Susan Radka. UK8.1 [235], 7.3.19.1 [236], PL3 [237], JS-1 [250] and MAD88 were obtained through the 10^{th} International

Histocompatibility Workshop. Optimal antibody concentrations and confirmation of antibody specificity were determined as previously described (Chapter 2) [248]. Tumors were classified based on tumor cell HLA-DR expression as $DR\beta^{-/-}$ (HLA-DR not expressed), $DR\beta^{-/+}$ (HLA-DRβ allotypes differentially expressed) or $DR\beta^{+/+}$ (HLA-DRβ allotypes homogeneously expressed).

3.3.4 RNA extraction, and semi quantitative RT-PCR

Total RNA was isolated from breast tumor tissues by homogenization in Trizol reagent (Gibco BRL, Rockville, MD), followed by treatment with DNA-free reagent (Ambion, Austin, TX) to remove any contaminating DNA. Reverse transcription was performed on 1 μg RNA using the First Strand cDNA Synthesis Kit (Pharmacia Biotech, Quebec, Canada). PCR was performed using a Biometra T Gradient thermocycler (Montreal Biotech Inc., Quebec, Canada) to amplify cDNA using primers described in Supplementary Table 3S2. Primer pairs specific for β-actin [251], IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-12 p40, TNF-α [252], IL-1β [253], TGF-β1 [254] and FOX-P3 [255] were synthesized by Gibco BRL. Primers were used at concentrations of 20 pM for β-actin, IL-1β, IL-6, TNF-α and FOX-P3 and 10 pM for all others. $MgCl_2$ (Gibco BRL) concentration was 1.5 mM for β-actin, IL-4 and FOX-P3 and 2mM for all other reactions. 0.2 μL of Taq DNA polymerase (Gibco BRL) was used for β-actin, IL-1β, IL-6 and FOX-P3 reactions and all others used 0.25 μL per reaction. All PCR reactions were performed in a volume of 50 μL with 200 μM dNTPs (Gibco BRL) and 1 μL cDNA. PCR buffer contained 20 mM Tris-HCl buffer (pH 8.4) and 50 mM KCl. Samples containing water instead of test cDNA were included as contamination controls and

cDNA from the cell lines Jurkat E6-1 (IL-2, IL-4, IL-10, TGF-β), C10/MJ (IFN-γ, IL-6, FOX-P3), U937 (TNF-α, IL-1β) and YAR (IL-12) were used as positive controls. Reaction mixtures were amplified for 35 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 55°C (IFN-γ and IL-2), 72°C (TNF-α) or 65°C (all others), and extension at 72°C for 1 min, followed by a separate 5 min extension step at 72°C for all but IL-1β, IL-6 and FOX-P3. The intensity of amplified products was semi-quantified and normalized as a percent of β-actin using the ChemiImager 4000 with Alphaease 4.0 Software.

3.3.5 Statistical Analysis

Contingency tables were analyzed using Pearson's chi-square test or Fisher's exact test for 2x2 tables when at least one expected count was ≤ 5 (two-sided). Non-parametric Mann-Whitney U test was used to assess statistical significance of all continuous variables except diagnosis age, which followed a normal distribution and was assessed by one-way analysis of variance (ANOVA). Variables were tested for normality using the Kolmogorov-Smirnov test. To assess HLA-DRβ allotypic influences, tumors were stratified only by those HLA-DRB allotypes that occurred at a frequency of at least 10%.

Survival estimates were calculated using Kaplan-Meier method with log-rank statistic. Ninety-five percent confidence intervals (95% CI) around each estimate were calculated using the standard error (SE) of the cumulative survival probability (95%CI = cumulative survival probability ±1.96 x SE). Estimates were calculated as time to distant metastasis for distant recurrence-free survival (dRFS) and time to death from breast

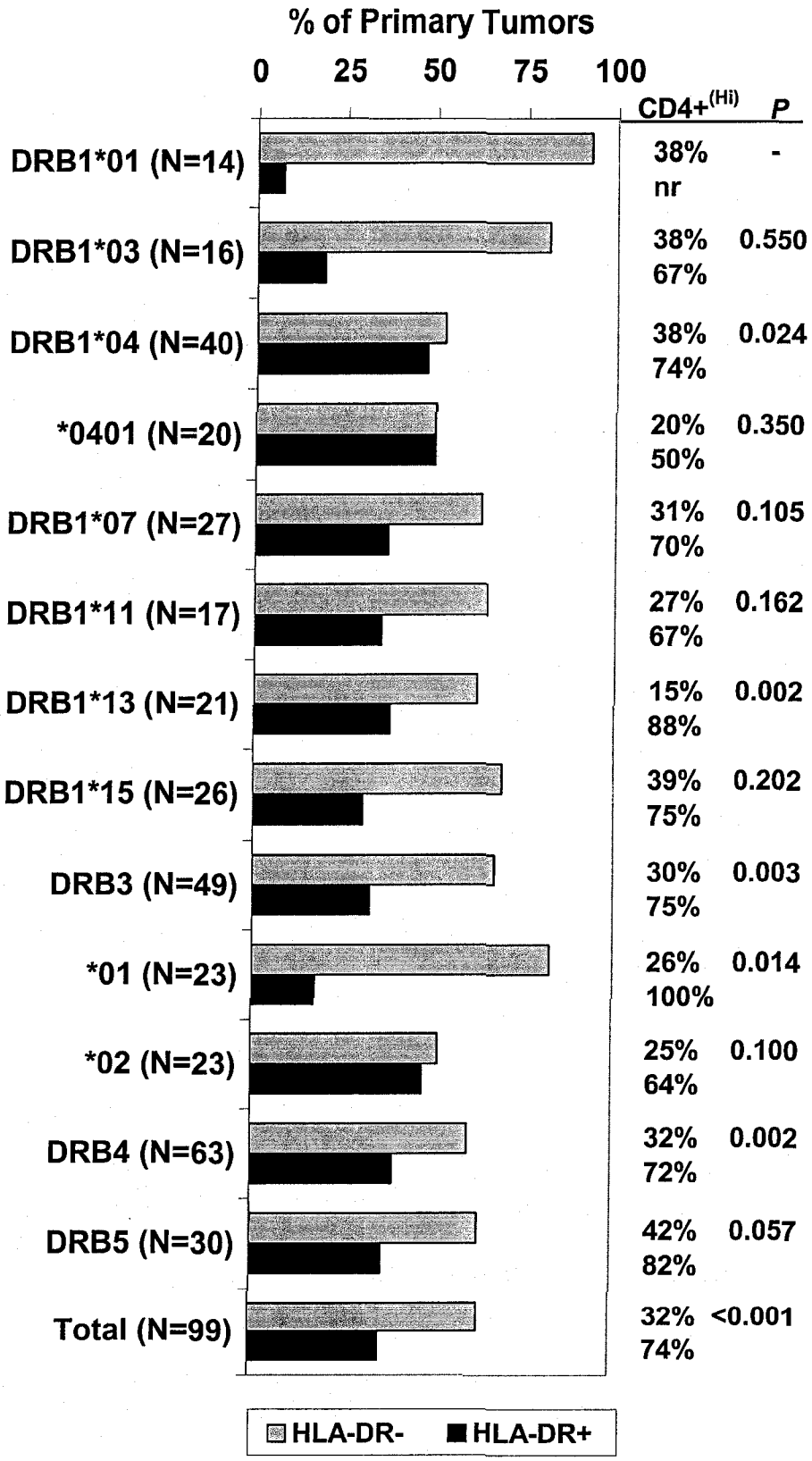
cancer for disease-specific survival (DSS). For DSS, patients who died of other causes were censored at the time of death. Cox proportional hazards models were constructed for multivariate survival analyses, using backward stepwise method, using a probability of $P<0.05$ for stepwise entry and $P>0.06$ for removal. No patients were lost to follow-up and patients not experiencing the event were censored at the time of last follow-up or at 5 years for 5-year survival. For multivariate analysis, correlation matrices were constructed to ensure lack of collinearity of covariates. All analysis was performed using SPSS Version 11.5 statistical software. All tests were two-sided and differences between groups were considered significant if $P<0.05$.

3.4 RESULTS

3.4.1 Characterization of tumor cell HLA-DR expression and associations with CD4+ TIL in breast carcinomas stratified by HLA-DRB allotypes

To ascertain whether genetic variation in HLA-DRB influences tumor cell expression of HLA-DR antigens and CD4+ TIL, primary tumors were stratified by HLA-DRB alleles. HLA-DR was expressed by tumor cells in 36/99 (36.4%) primary lesions. CD4+ TIL were detected intratumorally and throughout the intervening stroma in 82/97 (84.5%) breast tumors, with 46/97 (47.4%) containing moderate to large numbers of CD4+ TIL (CD4+^{Hi}). HLA-DR+ tumors were more frequently CD4+^{Hi} (Figure 3.1) and HLA-DR expression also associated with the pattern of CD4+ TIL infiltration. Of the 82 primary tumors that contained CD4+ TIL, T cells were diffusely distributed in 22/33 (66.7%) HLA-DR+ versus 21/49 (42.9%) HLA-DR- tumors ($\chi^2=4.5$, $P=0.034$).

Figure 3.1: HLA-DRB allotypic variation in tumor cell HLA-DR expression and associations with CD4+ TIL in breast carcinoma patients. HLA-DR expression was compared in primary tumors stratified by DRB allotypes. HLA-DRB1*04+ patients more frequently express HLA-DR antigens on their breast tumor cells (χ^2=3.6, *P*=0.058; as compared to non-DRB1*04 tumors). In contrast, HLA-DR is poorly expressed in tumors that carry DRB1*01 (χ^2=6.0, *P*=0.014), DRB1*03 (χ^2=2.6, *P*=0.110) and DRB3*01 (χ^2=5.4, *P*=0.020). Only HLA-DRB allotypes that occurred in at least 10% of patients are depicted. HLA-DR+ tumors more frequently contain CD4+ TIL (χ^2=15.8, *P*<0.001). This was observed in all HLA-DRB allotype groups. The percentage of tumors, in each group, with moderate to large numbers of CD4+ TIL (CD4+ ^{Hi}) is indicated at the right. nr = no result.


% of Primary Tumors
0
25
50
75
100
CD4+(Hi)
P
DRB1*01 (N=14)
38%
nr
-
DRB1*03 (N=16)
38%
67%
0.550
DRB1*04 (N=40)
38%
74%
0.024
*0401 (N=20)
20%
50%
0.350
DRB1*07 (N=27)
31%
70%
0.105
DRB1*11 (N=17)
27%
67%
0.162
DRB1*13 (N=21)
15%
88%
0.002
DRB1*15 (N=26)
39%
75%
0.202
DRB3 (N=49)
30%
75%
0.003
*01 (N=23)
26%
100%
0.014
*02 (N=23)
25%
64%
0.100
DRB4 (N=63)
32%
72%
0.002
DRB5 (N=30)
42%
82%
0.057
Total (N=99)
32%
74%
<0.001
HLA-DR-
HLA-DR+

In agreement with our previous study (Chapter 2) [248], tumors carrying DRB1*04 were more frequently HLA-DR+ than non-DRB1*04 tumors, reflected by the DRB1*0401 allele, although differences were not statistically significant. In contrast, HLA-DR expression was decreased in DRB1*01+, DRB1*03+ and DRB3*01+ tumors (Figure 3.1). Carriage of particular HLA-DRB alleles did not associate with the presence of CD4+ TIL (data not shown). However, tumors that expressed HLA-DR more frequently contained CD4+ TIL than HLA-DR- tumors, in all HLA-DRB allotype groups (Figure 3.1). The proportion of $CD4+^{Hi}$ HLA-DR+ tumors was decreased in DRB1*0401 tumors as compared to non-DRB1*0401 tumors that expressed HLA-DR (Fisher's exact test P=0.081) and within the $CD4+^{Lo}$ tumor subset, DRB1*0401+ tumors were more frequently HLA-DR+ [5/13 (38.5%) DRB1*0401+ versus 4/38 (10.5%) DRB1*0401-, Fisher's exact test P=0.036].

3.4.2 HLA-DRβ allelic variation affects associations of tumor cell HLA-DR expression with prognostic parameters in breast carcinoma patients

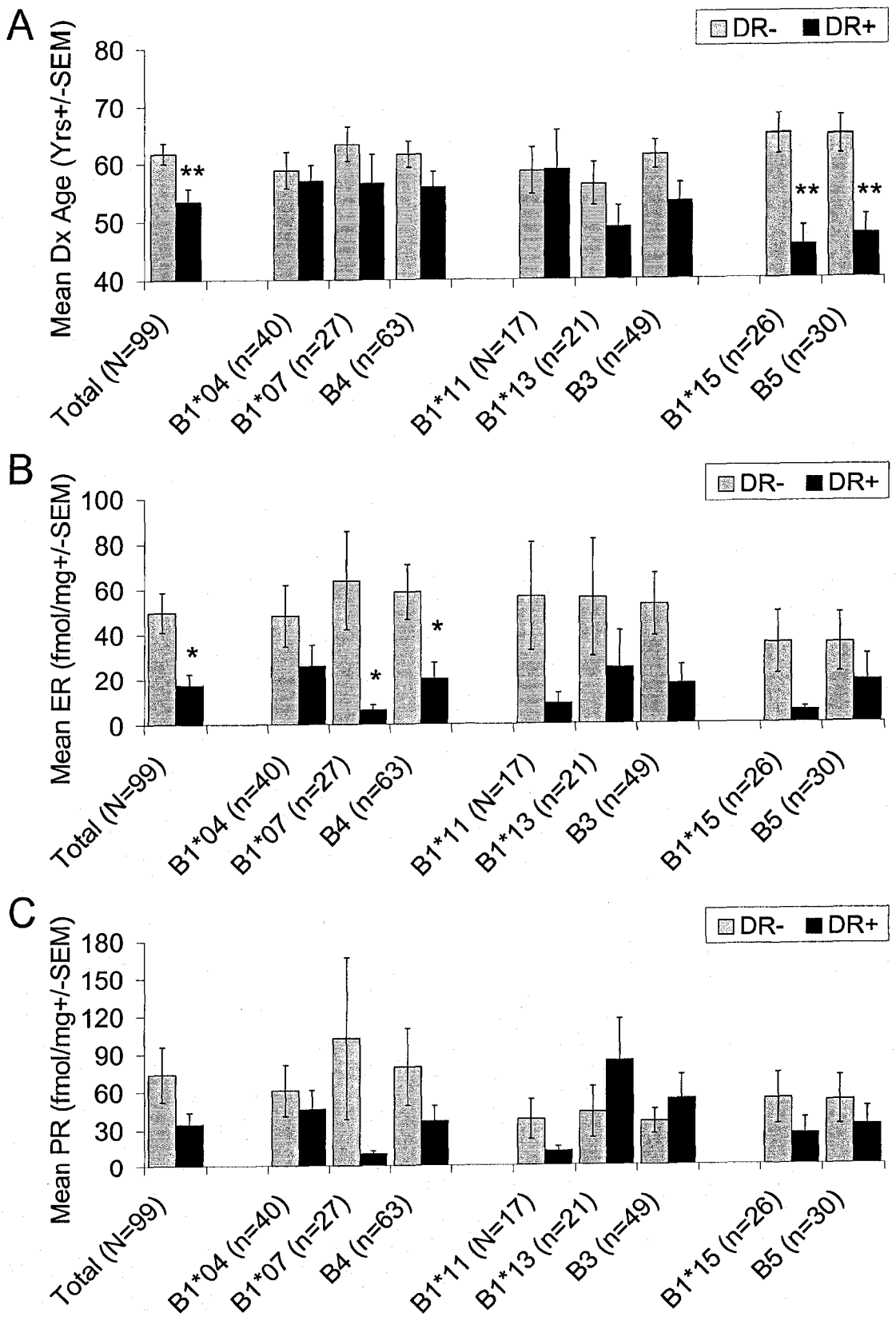
We have recently demonstrated that tumor cell expression of generic HLA-DR and the co-chaperone invariant chain associates with earlier age at diagnosis and reduced ER expression in breast carcinoma (Chapter 4) [256]. To determine whether associations of tumor cell HLA-DR expression with clinicopathological parameters are affected by HLA-DR alleles, we assessed these relationships in HLA-DR allotype groups. As HLA-DR was poorly expressed in DRB1*01+, DRB1*03+, and DRB3*01+ tumors, stratification was not warranted for these alleles.

The most pronounced association of diagnosis age and ER with tumor cell HLA-DR expression was observed in HLA-DRB1*15+ and HLA-DRB1*07+ individuals,

respectively. The age at diagnosis was decreased in all HLA-DRB allotype groups except DRB1*04+, DRB1*0401+ and DRB1*11+ patients (Figure 3.2A). All HLA-DR$^+$ tumors had decreased ER, regardless of HLA-DRB alleles (Figure 3.2B). PR levels were also decreased in HLA-DR+ tumors but differences were not statistically significant (Figure 3.2C). As all individuals are heterozygous for HLA-DRB alleles, to ensure the relationships observed across all HLA-DRB allele groups were not attributable to dominant effects of these alleles, associations were compared in HLA-DRB1 genotype categories. All genotype categories displayed a negative relationship between HLA-DR expression and diagnosis age except DRB1*04+DRB1*15-, DRB1*0401+DRB1*15- and DRB1*11+DRB1*15- tumors and all genotype groups showed decreased ER in DR$^+$ tumors (data not shown).

Although generic HLA-DR expression did not associate with tumor size or LN status, DRB allelic differences were observed (Figure 3.2D-E). Tumor diameter was decreased in DR+DRB1*04+ tumors (Figure 3.2D), reflected by the DRB1*0401 allotype group (P=0.065, data not shown). DR+DRβ1*13+ tumors had increased tumor diameter (Figure 3.2D) and were more frequently LN+ (Figure 3.2E), reflected by the associated DRB3 allotype group, which included DRB3*01, 02 and 03 alleles, all of which were represented in DRB1*13+ patients. The association was not significant for the DRB3*02 allele group, owing to the fact that both HLA-DR+ (10/11) and HLA-DR- (8/12) DRB3*02+ tumors are more frequently LN+ (χ^2=2.0, P=0.317). Tumor cell HLA-DR expression did not associate with tumor type, histological grade, TNM stage or Her-2/neu over-expression overall or within DRB allele groups.

Figure 3.2: Associations of tumor cell HLA-DR expression with prognostic parameters in breast carcinoma patients stratified by carriage of HLA-DRB allotypes. **A)** Diagnosis age is decreased in patients with HLA-DR+ tumors (F=8.0, *P*=0.006). This was most evident in DRB1*15+ patients (F=11.2, *P*=0.003), which also carry the DRB5 allotype (F=11.5, *P*=0.002) but was not observed in DRB1*04+ or DRB1*11+ individuals. **B)** ER levels are decreased in HLA-DR+ tumors (Mann-Whitney U test *P*=0.012). This was observed in all DRB allotype groups but was most evident in DRB1*07+ individuals (Mann-Whitney U test *P*=0.035), reflected by the linked DRB4 allotype (Mann-Whitney U test *P*=0.018). **C)** HLA-DR+ tumors also display a trend for decreased PR levels (Mann-Whitney U test *P*=0.071), although differences were not statistically significant within HLA-DRB allotype groups. **D)** HLA-DR expression associates with increased tumor diameter in DRB1*13+ patients (Mann Whitney U test *P*=0.012), which also carry the DRB3 allotype (Mann Whitney U test *P*=0.068), while DRB1*04+ patients have decreased tumor size when HLA-DR antigens are expressed (Mann Whitney U test *P*=0.065). **E)** HLA-DR expression associates with lymph node metastasis in DRB1*13+ (Fisher's exact test *P*=0.044) and DRB3+ (Pearson's χ^2=7.9, *P*=0.005) patients. **P*<0.05, ***P*<0.01.


A
DR-
DR+
Mean Dx Age (Yrs+/-SEM)
80
70
60
50
40
**
**
**
Total (N=99)
B1*04 (n=40)
B1*07 (n=27)
B4 (n=63)
B1*11 (N=17)
B1*13 (n=21)
B3 (n=49)
B1*15 (n=26)
B5 (n=30)
B
DR-
DR+
Mean ER (fmol/mg+/-SEM)
100
80
60
40
20
0
*
*
*
Total (N=99)
B1*04 (n=40)
B1*07 (n=27)
B4 (n=63)
B1*11 (N=17)
B1*13 (n=21)
B3 (n=49)
B1*15 (n=26)
B5 (n=30)
C
DR-
DR+
Mean PR (fmol/mg+/-SEM)
180
150
120
90
60
30
0
Total (N=99)
B1*04 (n=40)
B1*07 (n=27)
B4 (n=63)
B1*11 (N=17)
B1*13 (n=21)
B3 (n=49)
B1*15 (n=26)
B5 (n=30)

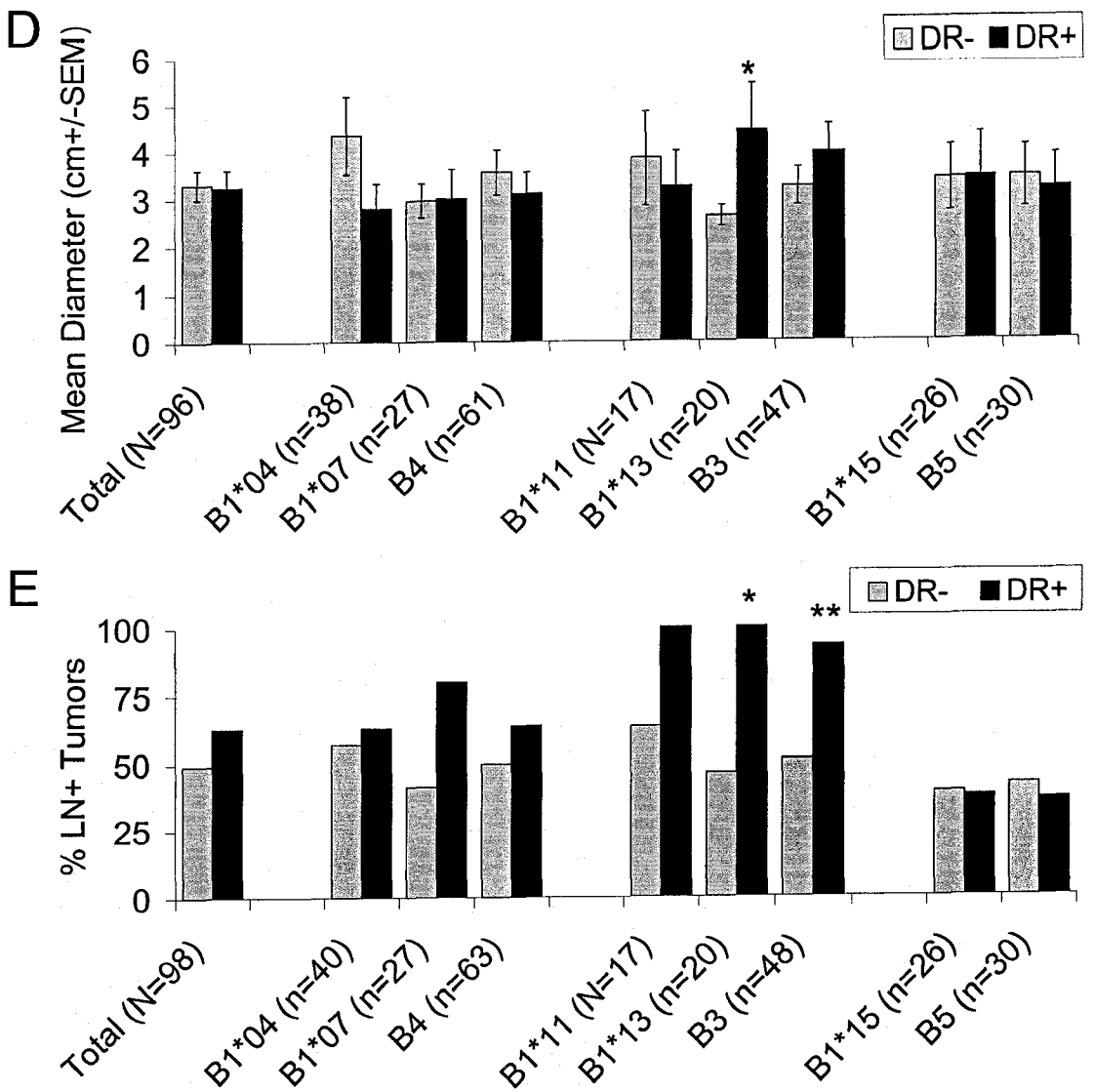


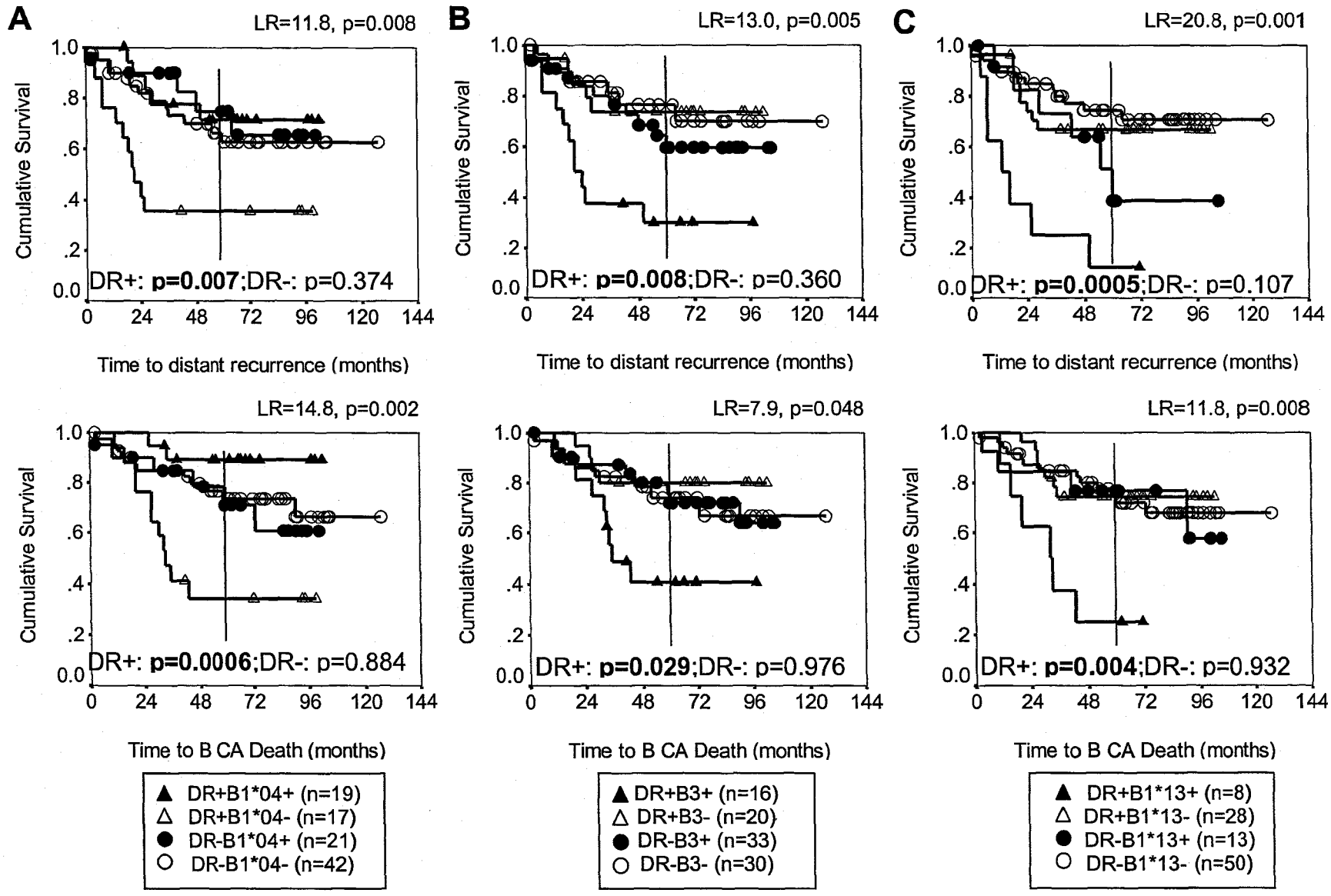
Figure 3.2 continued.

3.4.3 HLA-DRβ allelic variation affects associations of tumor cell HLA-DR expression with survival in breast carcinoma patients

Despite the associations of tumor cell HLA-DR expression with markers of a poor prognosis (Figure 3.2), univariate analysis did not reveal an influence on distant recurrence-free survival (dRFS; Log rank = 2.0, P=0.161) or disease-specific survival (DSS; Log rank = 0.7, P=0.393). We recently demonstrated patient survival is influenced by the HLA-DRB allelic differences (Drover et al., manuscript in preparation). In this study, survival rates were compared in tumors stratified by tumor cell HLA-DR expression and HLA-DRB alleles that occurred at a frequency of at least 10%, to ascertain whether the HLA-DRB alleles carried by the patient influence associations of tumor cell HLA-DR expression with patient survival. Within HLA-DR+ tumors, those that carried the DRB1*04 allotype had significantly improved dRFS and DSS (Figure 3.3A). In contrast, DR+DRB3+ tumors had decreased dRFS and DSS (Figure 3.3B). This was most evident in those patients that carried the HLA-DRB1*13 allele. DRB1*13+ tumors that did not express DR also displayed a trend for decreased dRFS but not DSS (Figure 3.3C). The stratification variables of the most frequent HLA-DRB1*04 allele, HLA-DRB1*0401 and of the HLA-DRB3 allele, HLA-DRB3*02, displayed similar patterns as DRB1*04 and DRB3 expression categories, respectively (data not shown). All other DRB stratification categories did not significantly associate with patient survival (data not shown).

Cox proportional hazards models were constructed to determine if the influence of HLA-DRB alleles on tumor cell HLA-DR expression independently predicted patient

Figure 3.3: The HLA-DRB allotypes carried by the patient influence associations of tumor cell expression of HLA-DR with distant recurrence-free survival (dRFS) and disease-specific survival (DSS) in breast carcinoma patients. **A)** Patients with DRB1*04+ tumors that express HLA-DR have improved dRFS and DSS as compared to patients with HLA-DR+ tumors that do not carry the DRB1*04 allotype (DR+DRB1*04-: #events/#cases = 11/17, 5-year % survival (95% CI) = 35(13-58) versus DR+DRB1*04+: 5/19, 72(51-93) for dRFS; DR+DRB1*04-: 11/17, 34(11-57) versus DR+DRB1*04+: 2/19, 89(75-100) for DSS). **B)** Patients with DRB3+ tumors that express HLA-DR have decreased dRFS and DSS as compared to patients with HLA-DR+ tumors that do not carry the DRB3 allotype (DR+DRB3-: 5/20, 74(54-94) versus DR+DRB3+: 11/16, 30(7-53) for dRFS; DR+DRB3-: 4/20, 80(63-98) versus DR+DRB3+: 9/16, 41(15-66) for DSS). **C)** Patients with DRB1*13+ tumors that express HLA-DR have decreased dRFS and DSS as compared to patients with HLA-DR+ tumors that do not carry the DRB1*13 allotype (DR+DRB1*13-: 9/28, 67(49-85) versus DR+DRB1*13+: 7/8, 13(0-35) for dRFS; DR+DRB1*13-: 7/28, 75(58-91) versus DR+DRB1*13+: 6/8, 25(0-55) for DSS). All other HLA-DRB allotypes did not show significant differences with respect to patient survival. Survival rates were compared using Kaplan-Meier method with Log rank (LR) statistic. Overall significance between the four groups is provided in top right corner, levels of significance for comparison of 5-year survival for stratified variables are indicated on graphs. Censored observations indicated by symbols.


A
LR=11.8, p=0.008
Cumulative Survival
1.0
.8
.6
.4
.2
0.0
DR+: p=0.007;DR-: p=0.374
0 24 48 72 96 120 144
Time to distant recurrence (months)
B
LR=13.0, p=0.005
DR+: p=0.008;DR-: p=0.360
Time to distant recurrence (months)
C
LR=20.8, p=0.001
DR+: p=0.0005;DR-: p=0.107
Time to distant recurrence (months)
LR=14.8, p=0.002
DR+: p=0.0006;DR-: p=0.884
Time to B CA Death (months)
▲ DR+B1*04+ (n=19)
△ DR+B1*04- (n=17)
● DR-B1*04+ (n=21)
○ DR-B1*04- (n=42)
LR=7.9, p=0.048
DR+: p=0.029;DR-: p=0.976
Time to B CA Death (months)
▲ DR+B3+ (n=16)
△ DR+B3- (n=20)
● DR-B3+ (n=33)
○ DR-B3- (n=30)
LR=11.8, p=0.008
DR+: p=0.004;DR-: p=0.932
Time to B CA Death (months)
▲ DR+B1*13+ (n=8)
△ DR+B1*13- (n=28)
● DR-B1*13+ (n=13)
○ DR-B1*13- (n=50)

survival. HLA-DRB allele/HLA-DR expression stratification variables were independently entered into Cox regression models that controlled for treatment, tumor type, TNM stage, estrogen receptor, progesterone receptor, and age at diagnosis. Histological grade and Her-2/neu status were not included in the models as their inclusion decreased sample size and did not alter the prognostic significance of the other covariates (data not shown). When controlling for the above prognostic factors, the DRB1*04/DR category was an independent predictor of dRFS and DSS, while DRB3/DR and DRB1*13/DR categories independently predicted dRFS but not DSS (Table 3.1). In particular, DRB1*04+ patients with HLA-DR+ tumors were only 0.24 times as likely to suffer a distant recurrence and 0.12 times as likely to die from breast cancer as compared to non-DRB1*04 patients with DR-expressing tumors. In contrast, DRB3+ patients with HLA-DR+ tumors were 4.47 times more likely to suffer a distant recurrence than non-DRB3 patients with HLA-DR+ tumors. Likewise, DRB1*13+ patients with HLA-DR+ tumors were 4.72 times more likely to suffer a distant recurrence than non-DRB1*13 patients with HLA-DR+ tumors. Similar results were obtained for 5-year survival. All other DRB allele stratification variables did not independently associate with patient outcome (data not shown).

3.4.4 Decreased survival of patients with HLA-DR+ tumors that differentially express HLA-DRβ allotypes is dependent on the HLA-DRB alleles carried by the patient

In a previous study we showed that most HLA-DR+ breast tumors differentially express individual HLA-DRβ allotypes [248], but we did not address its implications for prognosis and survivial. As stratification of tumors based on HLA-DRB alleles and

Table 3.1: Multivariate Cox Proportional Hazards regression models using backward stepwise method† to assess the significance of HLA-DRB alleles and tumor cell HLA-DR expression on patient survival.

Model	dRFS			DSS		
	Total			Total		
	p	HR	(95%CI)	p	HR	(95%CI)
1)DRB1*04/DR	**0.017**	-	-	**0.032**	-	-
DRB1*04-DR+	*Reference*			*Reference*		
DRB1*04+DR+	**0.011**	0.24	(0.08-0.73)	**0.006**	0.12	(0.02-0.55)
DRB1*04-DR-	**0.023**	0.37	(0.16-0.87)	0.067	0.41	(0.16-1.06)
DRB1*04+DR-	**0.010**	0.21	(0.06-0.69)	0.393	0.62	(0.21-1.85)
Model χ^2 (*P*-value)‡	20.1 (0.001)			20.8 (0.001)		
2) DRB3/DR	**0.020**	-	-	ns	-	-
DRB3-DR+	*Reference*			*Reference*		
DRB3-DR-	0.793	1.17	(0.36-3.85)	ns	-	-
DRB3+DR+	**0.008**	4.47	(1.49-13.44)	ns	-	-
DRB3+DR-	0.211	1.97	(0.68-5.68)	ns	-	-
Model χ^2 (*P*-value)	19.4 (0.002)			7.0 (0.008)		
3)DRB1*13/DR	**0.003**	-	-	ns	-	-
DRB1*13-DR+	*Reference*			*Reference*		
DRB1*13-DR-	0.556	0.76	(0.31-1.89)	ns	-	-
DRB1*13+DR+	**0.005**	4.72	(1.59-14.02)	ns	-	-
DRB1*13+DR-	0.133	2.25	(0.78-6.49)	ns	-	-
Model χ^2 (*P*-value)	28.2 (<0.001)			7.0 (0.008)		
# events/# at risk	32/91			28/91		

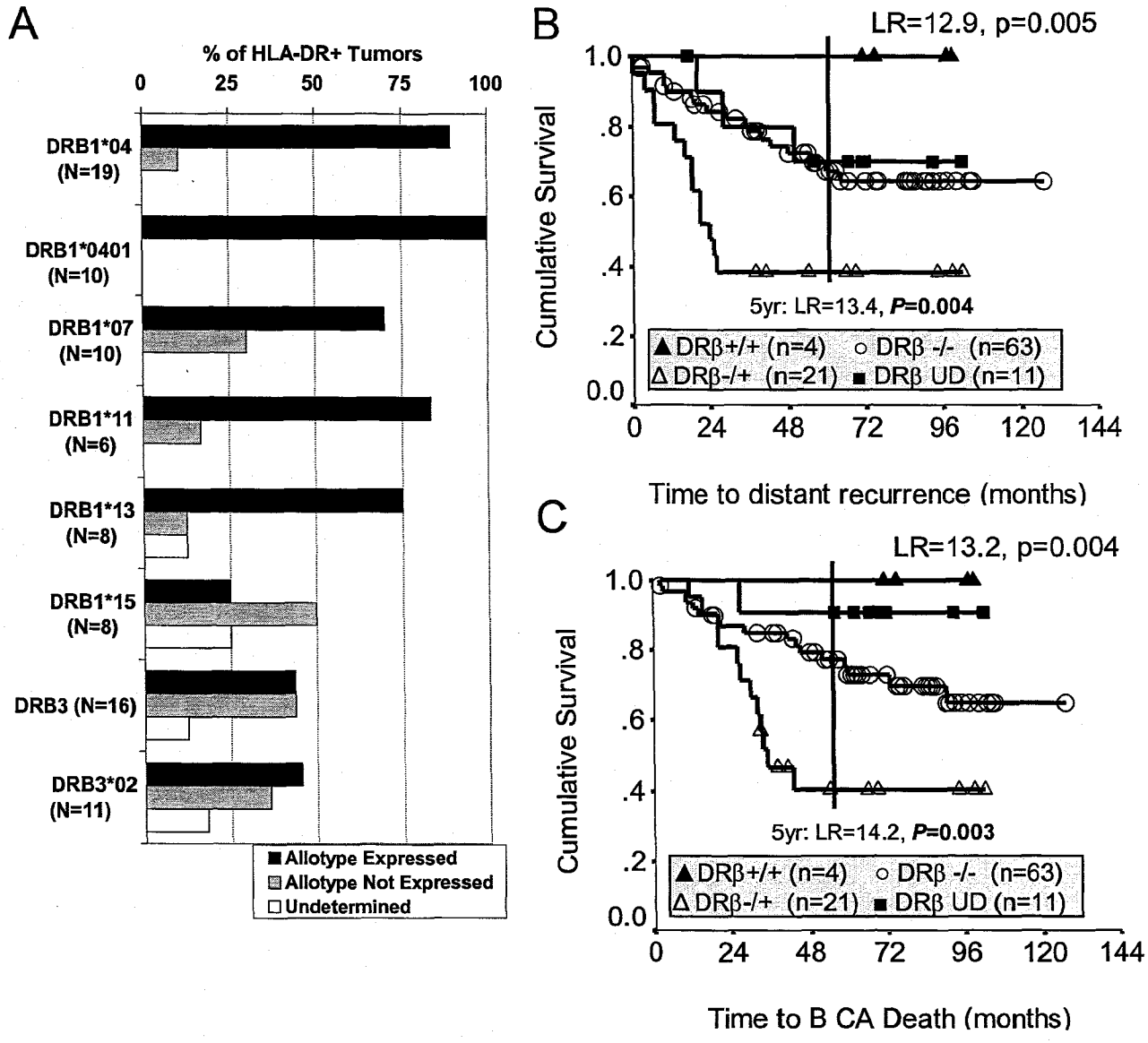
†Cox proportional hazards models were analyzed using backward stepwise method with entry at $P<0.05$ and removal at $P\geq0.06$. All models controlled for treatment, tumor type, TNM stage, diagnosis age, ER, and PR. For model 1, TNM stage and ER status were also independent predictors of dRFS, while TNM stage and PR independently predicted DSS. For model 2, ER status independently predicted dRFS while PR status was an independent predictor of DSS. For Model 3, TNM stage and ER status were also independent predictors of dRFS, while PR independently predicted DSS. HR=hazards ratio; CI=confidence interval for HR.

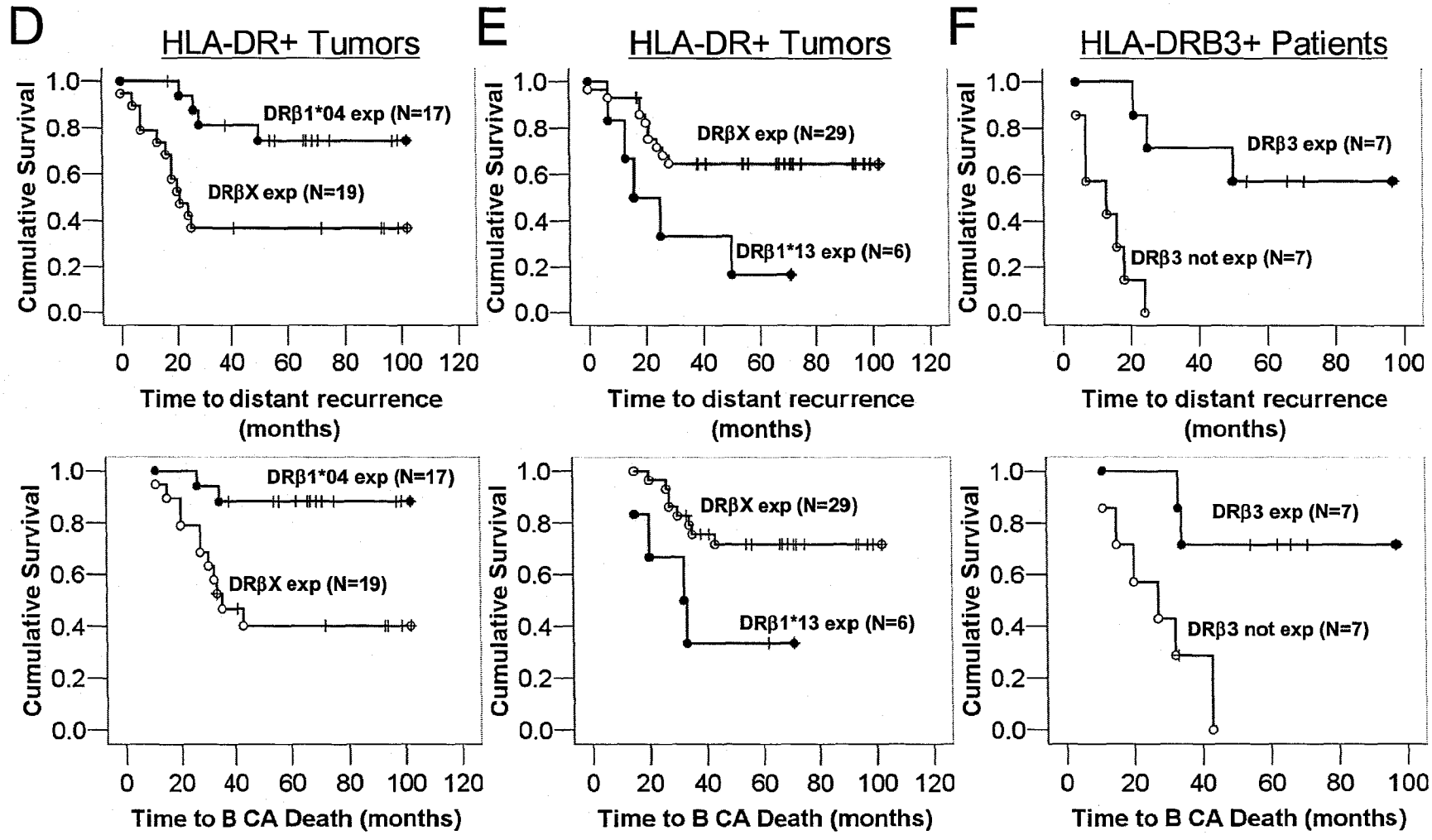
‡ Model statistics

generic tumor cell HLA-DR expression does not address individual HLA-DRβ allotype expression by tumor cells, we further assessed HLA-DRβ allotypic expression in HLA-DR+ primary tumors. A smaller numbers of recurrent breast carcinoma lesions were also examined to determine if differential HLA-DRβ allotype expression associates with disease progression. Using the available panel of allotype-discriminating antibodies, we determined at least 21/35 primary and 4/4 recurrent HLA-DR+ tumors lacked expression of one or more HLA- DRβ allotypes (Supplementary Table 3S3), thus confirming our previous findings. Representative examples of tumors that homogeneously or differentially express their HLA-DRβ allotypes are provided in Supplementary Figures 3S1 and 3S2, respectively. Discordant allelic expression was unique to the individual tumor as each of the allotypes not expressed was detected within another HLA-DR+ tumor (Supplementary Table 3S3). However, the DRβ1*04 allotype was expressed most frequently as 17/19 (89.5%) of DRβ1*04+ tumors expressed the DRβ1*04 allotype as compared to 27/42 (64.3%) of other DRβ1 allotypes. Furthermore, in the subgroup of patients that carry HLA-DRB3, tumor cell expression of the HLA-DRβ3 allotype could be determined in 14/16 HLA-DR+ tumors and notably 7/14 did not express the DRβ3 allotype (Figure 3.4A).

To ascertain whether DRβ allotypic expression by tumor cells has prognostic implications, prognostic parameters were assessed in tumors grouped based on expression of individual HLA-DRβ allotypes (Supplementary Table 3S4). As no particular HLA-DRβ allotype showed preferential dysregulation, associations of HLA-DRβ allotype expression categories with prognostic parameters paralleled that of generic HLA-DR expression, stratified by HLA-DRB alleles (Figure 3.2). Namely, diagnosis age

Figure 3.4: Differential expression of HLA-DRβ allotypes associates with decreased survival in breast carcinoma patients. **A)** Expression of individual HLA-DRβ allotypes in HLA-DR+ tumors was determined using indirect immunohistochemistry and a panel of allotype-specific monoclonal antibodies. **B-C)** Differential expression of HLA-DRβ allotypes by breast tumor cells associates with decreased dRFS and DSS. Survival rates were compared using Kaplan-Meier method with Log rank (LR) statistic. Overall significance between the four groups is provided in top right corner, levels of significance for 5-year survival rates are indicated on graphs. **D-E)** In the subset of HLA-DR+ tumors, expression of the HLA-DRβ1*04 allotype by breast tumor cells associates with improved dRFS (LR=7.3, P=0.007) and DSS (LR=8.5, P=0.004), while expression of the HLA-DRβ1*13 allotype associates with decreased dRFS (LR=5.9, P=0.016) and DSS (LR=4.9, P=0.028). **F)** Within the subset of patients that carry the HLA-DRB3 allotype and have HLA-DR+ tumor cells, dysregulated expression of the HLA-DRβ3 allotype associates with decreased dRFS (LR=12.6, P<0.001) and DSS (LR=7.3, P<0.001).


A
% of HLA-DR+ Tumors
0
25
50
75
100
DRB1*04 (N=19)
DRB1*0401 (N=10)
DRB1*07 (N=10)
DRB1*11 (N=6)
DRB1*13 (N=8)
DRB1*15 (N=8)
DRB3 (N=16)
DRB3*02 (N=11)
Allotype Expressed
Allotype Not Expressed
Undetermined
B
LR=12.9, p=0.005
Cumulative Survival
1.0
.8
.6
.4
.2
0.0
5yr: LR=13.4, P=0.004
▲DRβ+/+ (n=4) ○ DRβ -/- (n=63)
ΔDRβ-/+ (n=21) ■ DRβ UD (n=11)
0
24
48
72
96
120
144
Time to distant recurrence (months)
C
LR=13.2, p=0.004
Cumulative Survival
1.0
.8
.6
.4
.2
0.0
5yr: LR=14.2, P=0.003
▲DRβ+/+ (n=4) ○ DRβ -/- (n=63)
ΔDRβ-/+ (n=21) ■ DRβ UD (n=11)
0
24
48
72
96
120
144
Time to B CA Death (months)


D
HLA-DR+ Tumors
Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
DRβ1*04 exp (N=17)
DRβX exp (N=19)
0 20 40 60 80 100 120
Time to distant recurrence (months)
E
HLA-DR+ Tumors
DRβX exp (N=29)
DRβ1*13 exp (N=6)
F
HLA-DRB3+ Patients
DRβ3 exp (N=7)
DRβ3 not exp (N=7)
0 20 40 60 80 100
Time to B CA Death (months)

was decreased in all HLA-DRβ allotype expression categories except DRβ1*04, DRβ1*0401 and DRβ1*11 and ER was significantly decreased in all HLA-DRβ allotype expression categories as compared to $DR\beta^{-/-}$ tumors, but was most marked in tumors that express the DRβ1*07 allotype. PR was generally decreased in HLA-DRβ allotype expression categories, except in tumors that express the DRβ1*13 and/or DRβ3 allotypes. Tumors that express the DRβ1*0401 allotype have significantly decreased tumor diameter and all DRB1*13 and DRB1*11 patients with tumors that express the respective HLA-DRβ allotypes have LN metastasis.

To determine if differential expression of DRβ allotypes by tumor cells has clinical implications, univariate survival analysis was performed in tumors stratified by HLA-DRβ expression categories. Patients with $DR\beta^{-/+}$ tumors had markedly decreased dRFS and DSS (Figure 3.4B-C). Overall, differential DRβ allotype expression independently predicted dRFS and DSS by multivariate Cox proportional hazards regression analysis, controlling for standard prognostic indicators, as above. Using patients with $DR\beta^{-/-}$ tumors as a reference, tumors that differentially express HLA-DRβ allotypes ($DR\beta^{-/+}$) are 2.5 times more likely to suffer a distant recurrence and 2.3 times as likely to die from the disease within 5 years. Similar results were obtained when comparing overall survival rates (Supplementary Table 3S5).

Since stratification by generic HLA-DR expression and HLA-DRB alleles demonstrated allelic differences and tumor cells differentially express HLA-DRβ allotypes, we assessed the specific influence of HLA-DRβ allotype expression by comparing tumors in which a particular HLA-DRβ allotype was expressed to tumors that

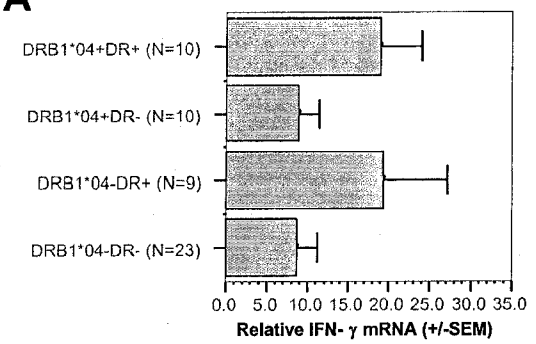
express other HLA-DRβ allotypes. Within HLA-DR+ tumors, those that express the DRβ1*04 allotype have improved survival, as compared to tumors that express other DRβ allotypes, while those that express the DRβ1*13 allotype have decreased survival (Figure 3.4D-E). Importantly, within HLA-DR+ tumors 17/19 DRB1*04 tumors express the HLA-DRB1*04 allotype and only 1/8 DRB1*13 tumors lack expression of the DRB1*13 allotype. Thus, similar results were obtained if only $DR\beta^{-/+}$ tumors were assessed, but with decreased numbers of samples (data not shown). Although the number of HLA-DR+ tumors that lack expression of HLA-DRβ1*04 or HLA-DRβ1*13 was too few to permit comparison with tumors that express these allotypes, DRβ3 was dysregulated in enough tumors to warrant further evaluation. Within HLA-DRB3 patients, lack of expression of the DRβ3 allotype by HLA-DR+ breast tumor cells associates with decreased dRFS and DSS (Figure 3.4F).

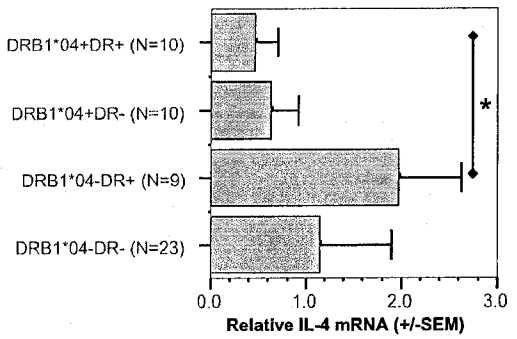
3.4.5 DRB1*13DR+ tumors have decreased IFN-γ and elevated IL-1β and IL-6 mRNA

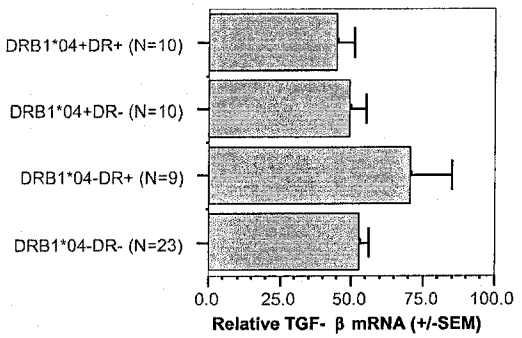
To determine if the inverse association with patient survival of DRB1*04 and DRB3 and DRB1*13 tumors that express HLA-DR was attributable to variation in immune responsiveness, cytokine mRNA levels were compared in HLA-DRB allotype subgroups stratified on HLA-DR expression (Figure 3.5). Stratification by HLA-DRB1*04 (Figure 3.5A) showed both HLA-DR+ subgroups have elevated IFN-γ. In contrast, HLA-DR+DRB1*04+ tumors have significantly lower IL-4 as compared to non-DRB1*04 tumors that express HLA-DR. HLA-DR+DRB1*04+ tumors also have markedly decreased TGF-β1 as compared to non-DRB1*04 HLA-DR+ tumors. This

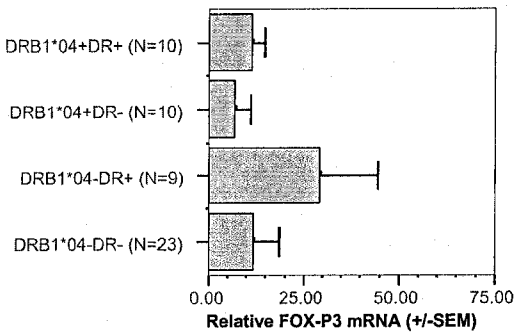
Figure 3.5: Relative intratumoral cytokine and FOX-P3 mRNA levels in breast tumors stratified by tumor cell HLA-DR expression and carriage of HLA-DRB allotypes. **A)** Stratification by HLA-DRB1*04 shows both HLA-DR+ tumor subsets have elevated IFN-γ mRNA (P=0.048), but non-HLA-DRB1*04 HLA-DR+ tumors have elevated IL-4 (P=0.079). Although not statistically significant, non-DRB1*04 HLA-DR+ tumors also have elevated TGF-β1 (P=0.578), FOX-P3 (P=0.101), and IL-1β (P=0.289). **B)** DRB3 HLA-DR+ tumors have decreased IFN-γ (P=0.033). IL-4 levels did not significantly differ (P=0.508) but relative levels of TGF-β1 (P=0.067), FOX-P3 (P=0.101), and IL-1β (P=0.126) were increased. **C)** DRB1*13 HLA-DR+ tumors have decreased IFN-γ (P=0.033). Relative levels of IL-4 did not significantly differ (P=0.409) but TGF-β1 (P=0.165), FOX-P3 (P=0.101), and IL-1β (P=0.022) were increased. Differences between the four groups were compared using Kruskal-Wallis H test. Asterisks indicate significant differences in HLA-DRB allotype groups within HLA-DR+ tumors, as determined by Mann-Whitney U test. *P<0.05.

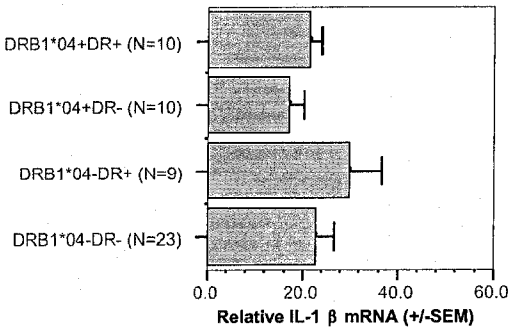
A


DRB1*04+DR+ (N=10)
DRB1*04+DR- (N=10)
DRB1*04-DR+ (N=9)
DRB1*04-DR- (N=23)
0.0 5.0 10.0 15.0 20.0 25.0 30.0 35.0
Relative IFN- γ mRNA (+/-SEM)


DRB1*04+DR+ (N=10)
DRB1*04+DR- (N=10)
DRB1*04-DR+ (N=9)
DRB1*04-DR- (N=23)
*
0.0 1.0 2.0 3.0
Relative IL-4 mRNA (+/-SEM)


DRB1*04+DR+ (N=10)
DRB1*04+DR- (N=10)
DRB1*04-DR+ (N=9)
DRB1*04-DR- (N=23)
0.0 25.0 50.0 75.0 100.0
Relative TGF- β mRNA (+/-SEM)


DRB1*04+DR+ (N=10)
DRB1*04+DR- (N=10)
DRB1*04-DR+ (N=9)
DRB1*04-DR- (N=23)
0.00 25.00 50.00 75.00
Relative FOX-P3 mRNA (+/-SEM)


DRB1*04+DR+ (N=10)
DRB1*04+DR- (N=10)
DRB1*04-DR+ (N=9)
DRB1*04-DR- (N=23)
0.0 20.0 40.0 60.0
Relative IL-1 β mRNA (+/-SEM)

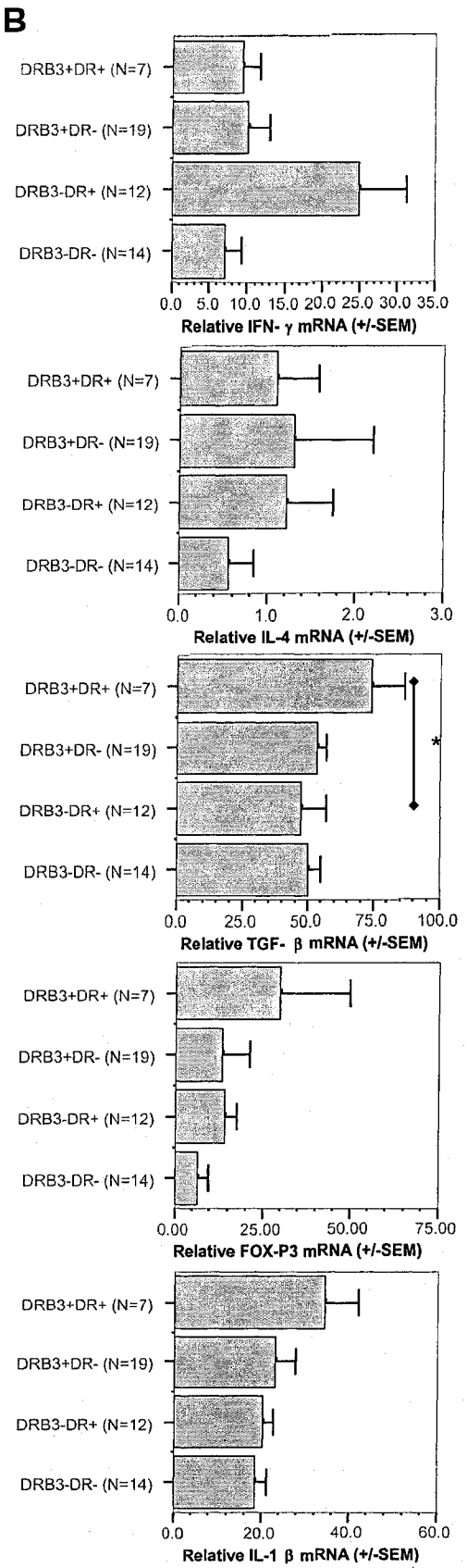


Figure 3.5 continued

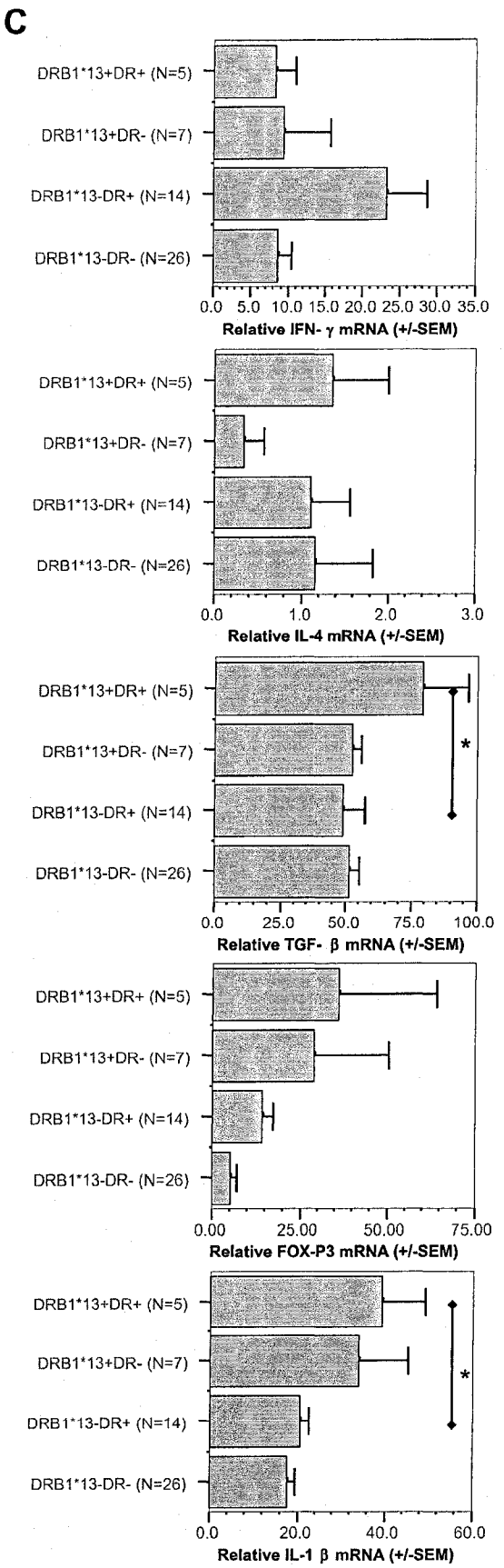


Figure 3.5 continued

trend was enhanced in the most frequent HLA-DRB1*04 allele, DRB1*0401 (Kruskal Wallis H test, P=0.052, data not shown), which also demonstrate a similar relationship as DRB1*04 with IFN-γ (Kruskal-Wallis H test, P=0.077) (data not shown). Non-DRB1*04 HLA-DR+ tumors also show a trend for increased FOX-P3 and IL-1β. These relationships were largely influenced by the DRB3 haplotype group, in particular DRB1*13+ tumors. Both DRB3 HLA-DR+ and DRB1*13 HLA-DR+ tumors contain decreased IFN-γ and elevated TGF-β1, FOX-P3 and IL-1β (Figure 3.5B-C). HLA-DR+ tumors that carry DRB3 and DRB1*13 also contain elevated levels of IL-6 [Kruskal-Wallis H test, P=0.016 (DRB3), P=0.049 (DRB1*13), data not shown]. Other cytokine mRNA levels did not significantly differ in DRB allotype categories (data not shown).

3.5 DISCUSSION

The results of this study suggest that the course of disease in breast carcinoma is influenced by allelic variation in HLA class II antigens, likely via effects on the anti-tumor immune response. Carriage of the HLA-DRB3 allele, associates with advanced disease and decreased survival in breast carcinoma patients (Drover et al., manuscript in preparation). This may in part be attributable to increased levels of the pro-inflammatory cytokines IL-1β and IL-6 in these patients. IL-1β and IL-6 can facilitate neoplastic progression via stimulation of cancer cell growth and production of pro-angiogenic factors, such as VEGF [257]. Levels of IL-1β were highest in DRB3 and DRB1*13 patients with HLA-DR expressing tumor cells. This likely reflects the ability of IL-1β to induce HLA-DR expression in breast carcinoma cells [135]. Furthermore, HLA-DRB3 patients with HLA-DR+ tumors, particularly those that carry the DRB1*13 allele, associate with the poor prognostic indicators of increased tumor diameter and lymph

node metastasis and independently predicted poor distant recurrence-free survival. Although the infiltrating CD4+ T cells were not phenotyped in this study, breast carcinomas do contain elevated numbers of CD4+ T regulatory cells [184]. Thus, HLA-DR expression by DRB1*13+ tumors may result in inefficient anti-tumor immunity via the activation of immunoregulatory CD4+ cells or T_H2 CD4+ T cells, as opposed to effector CD4+ T_H1 cells, as suggested by the decreased levels of IFN-γ mRNA and elevated TGF-β1 and FOX-P3 mRNA in DRB1*13 tumors that express HLA-DR. Recent studies have demonstrated HLA-DRB1*13 restricted TAA-specific T_{reg} can be generated from TIL of cancer patients following limiting dilution [258]. In addition, the ligands that preferentially stimulate TAA-specific CD4+ T_{reg} are antigens that are processed and presented by tumor cells but not pAPC pulsed with the same tumor cell lysates [259].

In support of our previous study [248], DRβ1*04 is expressed more frequently by tumor cells than other DRβ allotypes. HLA-DR expressing DRB1*04+ tumors displayed elevated IFN-γ and decreased TGF-β1. As TGF-β1 can suppress IFN-γ induction of HLA-DR via Smad3 dependent inhibition of CIITA promoter IV activity [260], decreased TGF-β1 in this subset of DRB1*04 tumors may allow IFN-γ mediated induction of HLA-DR antigens. However, although all 10 DR+DRB1*0401+ tumors expressed the DRβ1*0401 allotype, only 50% contained moderate to large numbers of infiltrating CD4+ T cells and it was these tumors that had elevated IFN-γ (data not shown). Furthermore, HLA-DR expression in DRB1*0401 tumors did not associate with decreased age at diagnosis, suggesting factors other than T-cell derived cytokines and hormones may induce HLA-DRβ1*0401 expression in DRB1*0401+ tumor cells. Using competitive RT-PCR, Vincent et al. (1996) [118] demonstrated that levels of DRβ1*04

mRNA in peripheral blood B cells were increased over predicted based on DRB1*04 promoter activity [117], suggesting a post-transcriptional modification. Reasons for enhanced expression of DRβ1*0401 are unknown but may involve differential targeting of HLA-DRβ1*0401 molecules through the endocytic pathway and differential peptide loading of DRβ1*0401 molecules, via heat shock protein interactions [261]. HLA-DR expression by DRB1*04+ tumors was an independent predictor of improved DSS. DRB1*0401 transfected neuroendocrine epithelial cells are known to express a wide pool of self-protein derived peptides from the plasma membrane and cytoplasm, as compared to DRB1*0401 homozygous B cell line [88]. Thus, DRB1*04+ patients may display an enhanced propensity for presentation of endogenous tumor antigen derived peptides by tumor cells and promote the activation of tumor-specific CD4+ T_H1 cells. In support of this, at the time of surgery, DRB1*04+DR+ tumors had a smaller tumor diameter and DRB1*04+ tumors that express HLA-DR in the presence of a CD4+ infiltrate have elevated IFN-γ mRNA levels. In contrast, DRB1*04+DR+ tumors have significantly decreased intratumoral levels of the T_H2 cytokine, IL-4 as compared to non-DRB1*04 HLA-DR+ tumors.

This study did not confirm the poor expression of HLA-DR in DRβ1*07+ tumors that we previously observed [248]. However, all DRβ1*07+ tumors that expressed DR had ER levels <10fmol/mg and in our previous study all DR-DRβ1*07+ tumors were ER+ (unpublished observations). The negative association of DR with ER expression supports the finding that 17-β estradiol down-modulates IFN-γ-induced MHC class II expression in a variety of cell types [262], and involves ER binding to MHC class II promoters [263]. Furthermore, 17-β estradiol also inhibits IL-1α and IL-1β induction of

HLA-DR in ER+ human endometrial and breast carcinoma cell lines in a dose dependent manner [135]. In this larger study, poor expression of DR by tumor cells was observed in tumors carrying the DRB1*01, DRB1*03 and DRB3*01 allotypes, despite the presence of CD4+ TIL. These tumor subgroups have later age at diagnosis and decreased ER levels, possibly explaining poor DR-expression (data not shown). However, the highest expression of HLA-DR antigens was observed in young women with ER- tumors (data not shown), suggesting in the absence of ER expression on tumor cells, the suppressive effects of E_2-ER complexes on HLA-DR induction may be overcome. The association with earlier age at diagnosis further suggests a role for circulating hormones in the induction of HLA-DR. These effects may be indirect as 17β-estradiol is known to enhance the expansion of antigen-specific CD4+ T_H1 cells and the production of IFN-γ *in vivo* [264]. Circulating estradiol also promotes the *in vivo* expansion of regulatory CD4+ T cells [265]. As such, in the absence of competing ER on breast tumor cells, estradiol may promote the expansion of tumor infiltrating CD4+ T cells, whose phenotype is dependent on the HLA-DRB alleles carried by the patient.

Loss of expression of individual HLA class I allospecificities occurs in breast carcinoma [224, 225] and experimental evidence suggests a role for T cell pressure in the generation of HLA loss variants [226, 227, 266]. We recently demonstrated this phenomenon also occurs for HLA-class II allotypes through the discovery of selective HLA-DRβ allospecificity expression in a small number of breast carcinoma lesions [248] and have confirmed this finding in this study. The proportion of $DR\beta^{-/+}$ tumors was increased in this subset of patients, possibly reflecting the biased selection and increased frequency of DRB1*04+ tumors in the previous study [248]. As differential DRβ allotype

expression is unique to an individual tumor, it supports the hypothesis that immune selective pressure will be dependent on both the HLA-DR genotype of the patient and the array of tumor-associated antigens expressed in that particular tumor allowing outgrowth of subpopulations of tumor cells that have lost the relevant HLA-DR antigens. In support of this, patients with primary tumors that differentially express DRβ allotypes have decreased dRFS and DSS and all metastatic lesions examined in this study were DR- or showed differential DRβ expression. Indeed, within individual patients, lymph node metastases are more frequently HLA class I and HLA class II negative than primary breast lesions [247].

As stratification of tumors based on HLA-DRB allele and generic tumor cell HLA-DR expression takes into account expression on pAPC and/or tumor cells but does not address individual HLA-DRβ allotype expression by tumor cells, we further assessed HLA-DRβ allotypic expression in HLA-DR+ primary tumors. Of the DRβ allotypes examined, DRβ3 most frequently demonstrated selective expression. Thus, in this subset of tumors, DRβ3 may represent a restricting element for TAA-specific effector CD4+ TIL and its expression may be downregulated in response to selective immune pressure. Although the number of samples analyzed is too few to warrant definitive conclusions, in support of this hypothesis, all DRB3 patients with HLA-DR+ tumors that fail to express the DRβ3 allotype suffered a distant recurrence within 2 years. In addition, since DRB3 is in linkage with HLA-DRB1*13, the poor survival in HLA-DRB1*13 patients with HLA-DR+ tumors, may in part be influenced by down-regulation of the DRβ3 allotype. Indeed 3/6 DRβ1*13 expressing tumors lack expression of the DRβ3 allotype (Supplementary Table 3S3).

The mechanisms responsible for differential DRβ allotypic expression have not yet been elucidated. One potential mechanism that may contribute to this phenomenon is loss of heterozygosity (LOH) at the HLA locus, as this mechanism causes HLA haplotype loss in a high percentage of tumors [238]. The pattern of allotypic expression in HLA-DR+ tumors, suggests LOH at chromosome 6p21.3 may contribute to selective HLA-DRβ expression in a subset of HLA-DR+ tumors, based on expression of DRβ1 allotypes and linked DRβ3, DRβ4 or DRβ5 allotypes (Supplementary Table 3S3). The pattern of allotypic expression in the HLA-DR+ tumors that differentially express HLA-DRβ allotypes, suggests that LOH may be responsible for differential DRβ allotype expression in 13/21 primary tumors and 4/4 recurrent tumors. Although HLA-DRβ$^{-/-}$ tumors were more frequently HLA class I-, the percentage of HLA class I- tumors did not significantly differ between DRβ$^{-/+}$ and DRβ$^{+/+}$ tumors [mean±SEM = 13.6±5.1% (DRβ$^{-/+}$); 5.0±5.0 (DRβ$^{+/+}$); P=0.695], nor did the percentage of tumor cells with weak HLA class I expression [mean±SEM = 15.2±6.3 (DRβ$^{-/+}$); 10.0±10.0% (DRβ$^{+/+}$); P=0.915], suggesting differential DRβ expression was not attributable to a dominant effect on the HLA locus, for the majority of HLA-DR+ tumors. Selective HLA class I antigen loss can occur due to mutations in the relevant HLA class I α chain, including large deletions and single base pair deletions or substitutions [229]. Similar defects in HLA-DRB alleles may contribute to the selective expression of HLA-DRβ allotypes in HLA-DR+ tumor cells.

The common occurrence of tumor cells with altered HLA expression and the influence of HLA genetics on tumor cell expression of HLA-DR antigens highlight the complexity of host-tumor interactions. Future studies aimed at thoroughly characterizing the immune response in breast carcinoma patients and correlating this with patterns of

HLA antigen expression and the genetics of the patient should provide valuable advances in the knowledge of which patients might benefit from immunotherapeutic intervention. Successful cancer immunotherapy will likely require the preferential activation of CD4+ T_H cells as opposed to T_{reg} cells. Such strategies will likely be unique to the patient and dependent on both the HLA-DRB alleles carried by the patient and the array of TAA expressed by the tumor cells.

Supplementary Table 3S1: DRB Allotype Specific mAbs used in this Study

mAb	Isotype	Concentration/ Dilution	DRβ Specificity
NFLD.D1	IgG1	10μg/ml	β1*04
NFLD.D7	IgG1	1/100	β1*04, 15, 16; β3
NFLD.D10	IgG1	3μg/ml	β1*01, 15, 04†, 14†, 09, 10; β5*02
SFR16	IgG2a	1/25	β1*07
UK8.1	IgG2b	1/1000	β1*03, 11, 13, 14†
7.3.19.1	IgG2b	1/1000	β1*03, β3
PL3	IgG3	1/400	β1*07, 09; β4
JS-1	IgG2a	1/1000	β1*01†, 03, 04†, 1402, β3
MAD88‡	IgG	1/100	β1*08, β3

† = Not all allelic products of this HLA-DR type carry the epitope recognized by this antibody

‡ = This mAb was reported as DRβ1*08 specific in the 10th IHW but shows cross reaction with additional DRβ3 allotypes.

Supplementary Table 3S2: Primer sequences for detection of mRNA in breast carcinoma lesions.

	Sense Primer (5'-3')	Anti-sense Primer (5'-3')	Size (bp)	GenBank Accession Number*
β-Actin	ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG	CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC	840	NM_005159
IFN-γ	AGT TAT ATC TTG GCT TTT CA	ACC GAA TAA TTA GTC AGC TT	356	J00219
IL-2	ACT CAC CAG GAT GCT CAC AT	AGG TAA TCC ATC TGT TCA GA	269	HSU25676
IL-12 p40	CCA AGA ACT TGC AGC TGA AG	TGG GTC TAT TCC GTT GTG TC	355	NM_002187
IL-4	CCT CTG TTC TTC CTG CTA GCA TGT GCC	CCA ACG TAC TCT GGT TGG CTT CCT TCA	371	M23442
IL-10	ATG CCC CAA GCT GAG AAC CAA GAC CCA	TCT CAA GGG GCT GGG TCA GCT ATC CCA	352	NM_000572
TGF-β	GCC CTG GAC ACC AAC TAT TGC	AGG CTC CAA ATG TAG GGG CAG G	165	X02812.1
FOX-P3	CAG CTG CCC ACA CTG CCC CTA G	CAT TTG CCA GCA GTG GGT AG	384	AF277993
IL-1β	ACA GAT GAA GTG CTC CTT CCA	GTC GGA GAT TCG TAG CTG GAT	75	BT007213
IL-6	AGC TCA GCT ATG AAC TCC TTC TC	GTC TCC TCA TTG AAT CCA GAT TGG	340	NM_000600
TNF-α	CGG GAC GTG GAG CTG GCC GAG GAG	CAC CAG CTG GTT ATC TCT CAG CTC	354	NM_000594

* All primer sequences were validated using published GenBank mRNA sequences.

Supplementary Table 3S3: HLA-DRβ allotype expression and CD4+ TIL in primary and recurrent breast tumors[†]

Sample ID	HLA-DR	HLA-DRβ Allotype Expression by Tumor Cells			CD4+ TIL	Distribution
		Expressed	Not Expressed	Undetermined		
11353p	+	β1*08, β1*16, β5	-	-	++	D
11452p	+	β1*0403, β1*11, β3*02, β4	-	-	++	D
12450p	+	β1*0406, β1*13, β3*03, β4	-	-	+	FD
12817p	+	β1*0401, β1*07, β4, β4	-	-	-/+	F
10810p	+	β1*0401, β1*0401[‡]	-	β4, β4	-/+	F
10881p	+	β1*0404, β1*13, β3*01	-	β4	+	FD
11070p	+	β1*0407	-	β1*15, β4, β5	++	FD
11937p	+	β1*07, β1*07	-	β4, β4	+	F
12291p	+	β1*0405, β1*07	-	β4, β4	++	FD
12483p	+	β1*07, β1*15, β4	-	β5	++	FD
12513p	+	β1*0401	-	β1*09, β4, β4	+	D
12816p	+	β1*0407	-	β1*16, β4, β5	+	FD
13058p	+	β1*15, β1*15	-	β5, β5	+	D
13412p	+	β1*0401, β1*0404	-	β4, β4	-	.
10867p	+	β1*1302, β1*1303	β3*01, β3*03	-	++	FD
11432p	+	β1*16	β1*1305, β3*02, β5	-	+	FD
13091p	+	β1*0401, β1*1101, β3*02	β4	-	-/+	F
13123p	+	β1*1302, β3*02, β3*03	β1*1401	-	+	FD
10903p	+	β1*1301	β1*15, β3*01	β5	++	D
10970p	+	β1*07	β1*08	β4	++	FD

Sample ID	HLA-DR	HLA-DRβ Allotype Expression by Tumor Cells			CD4+ TIL	Distribution
		Expressed	Not Expressed	Undetermined		
11008p	+	β1*1305	β3*02	β1*10	-/+	F
11129p	+	β1*0401	β4	β1*15, β5	+	D
11352p	+	β1*03	β3*01, β3*01	β1*1301	+	D
11673p	+	β1*07, β1*1101	β3*02	β4	+	FD
11889p	+	β1*0407, β3*02	β1*03	β4	+	F
12273p	+	β1*1104	β1*08	β3*02	+	FD
12716p	+	β1*07, β4	β1*15	β5	-/+	F
13088p	+	β1*0401, β3*02	β1*03	β4	-	-
13118p	+	β1*1104, β4	β1*07	β3*02	-/+	FD
13282p	+	β1*0401	β1*07	β4, β4	++	FD
13336p	+	β1*0401	β4	β1*10	+	F
13337p	+	β1*0401	β1*07	β4, β4	+	F
11225p	+	-	β1*0405, β1*15, β4	β5[§]	+	F
12418p	+	-	β1*08, β1*15	β5[§]	-/+	F
11644p	+	-	β1*01, β1*10[‖]	-	nr	
13067p	+	-	β1*0404, β1*1101, β3*02, β4[¶]	-	+	D
9571p	-	-	β1*0401, β1*11, β3, β4	-	-/+	F
9772p	-	-	β1*01, β1*08	-	-	-
10796p	-	-	β1*07, β1*07, β4, β4	-	-/+	FD
10814p	-	-	β1*01, β1*0401, β4	-	-	-
10832p	-	-	β1*03, β1*15, β3*01, β5	-	-/+	F
10850p	-	-	β1*1102, β1*13, β3*01, β3*02	-	-	-
10902p	-	-	β1*07, β1*15, β4, β5	-	-/+	F

Sample ID	HLA-DR	HLA-DRβ Allotype Expression by Tumor Cells			CD4+ TIL	Distribution
		Expressed	Not Expressed	Undetermined		
10958p	-	-	β1*01, β1*07, β4	-	-/+	FD
10960p	-	-	β1*0404, β1*0404, β4, β4	-	-/+	D
11051p	-	-	β1*01, β1*07, β4	-	+	D
11084p	-	-	β1*15, β1*15, β5, β5	-	+	FD
11089p	-	-	β1*03, β1*07, β3*01, β4	-	-/+	F
11090p	-	-	β1*1101, β1*1301, β3*01, β3*02	-	-/+	F
11108p	-	-	β1*04, β1*08, β4	-	+	FD
11125p	-	-	β1*0407, β1*15, β4, β5	-	-/+	F
11170p	-	-	β1*07, β1*1104, β3*02, β4	-	-	-
11208p	-	-	β1*0402, β1*15, β4, β5	-	-	-
11216p	-	-	β1*1301, β1*1303, β3*01, β3*02	-	-	-
11332p	-	-	β1*0401, β1*15, β4, β5	-	-/+	F
11372p	-	-	β1*07, β1*08, β4	-	nr	
11378p	-	-	β1*03, β1*03, β3*01, β3*01	-	+	D
11418p	-	-	β1*01, β1*03, β3*01	-	-/+	F
11555p	-	-	β1*0407, β1*08, β4	-	+	FD
11556p	-	-	β1*1104, β1*16, β3*02, β5	-	++	FD
11565p	-	-	β1*0407, β1*11, β3*02, β4	-	++	FD
11740p	-	-	β1*03, β1*13, β3, β3	-	-	-
11757p	-	-	β1*01, β1*1302, β3*03	-	+	F
11799p	-	-	β1*03, β1*1301, β3*01, β3*02	-	-/+	F
11806p	-	-	β1*0401, β1*0405, β4, β4	-	-/+	F
11818p	-	-	β1*03, β1*0404,	-	+	D

Sample ID	HLA-DR	HLA-DRβ Allotype Expression by Tumor Cells			CD4+ TIL	Distribution
		Expressed	Not Expressed	Undetermined		
			β3*01, β4			
11894p	-	-	β1*1201, β1*1301, β3, β3	-	-/+	F
11911p	-	-	β1*0404, β1*07, β4, β4	-	++	FD
11915p	-	-	β1*15, β1*15, β5, β5	-	-/+	F
12049p	-	-	β1*07, β1*07, β4, β4	-	-/+	FD
12078p	-	-	β1*01, β1*1101, β3*02	-	+	FD
12094p	-	-	β1*03, β1*0402, β3, β4	-	-/+	FD
12159p	-	-	β1*1101, β1*1104, β3*02, β3*02	-	-	-
12311p	-	-	β1*0401, β1*08, β4	-	-/+	F
12375p	-	-	β1*0103, β1*03, β3*01	-	+	FD
12495p	-	-	β1*07, β1*15, β4, β5	-	+	D
12511p	-	-	β1*01, β1*07, β4	-	-/+	F
12694p	-	-	β1*07, β1*1301, β3*01, β4	-	-	-
12748p	-	-	β1*1301, β1*15, β3*01, β5	-	-/+	F
12750p	-	-	β1*07, β1*1402, β3*01, β4	-	-/+	F
12782p	-	-	β1*1101, β1*1303, β3*01, β3*02	-	-/+	F
12831p	-	-	β1*03, β1*0401, β3*01, β4	-	+	F
12858p	-	-	β1*0401, β1*15, β4, β5	-	-/+	F
12926p	-	-	β1*03, β1*07, β3*01, β4	-	-	-
12937p	-	-	β1*07, β1*15, β4, β5	-	+	F
13054p	-	-	β1*01, β1*1302, β3*03	-	-	-
13089p	-	-	β1*03, β1*0404, β3*01, β4	-	+	FD
13113p	-	-	β1*1101, β1*14, β3*02, β3*02	-	-/+	D

Sample ID	HLA-DR	HLA-DRβ Allotype Expression by Tumor Cells			CD4+ TIL	Distribution
		Expressed	Not Expressed	Undetermined		
13269p	-	-	β1*09, β1*1101, β3*02, β4	-	-/+	F
13287p	-	-	β1*0401, β1*15, β4, β5	-	+	FD
13360p	-	-	β1*0408, β1*1303, β3*01, β4	-	-/+	F
13369p	-	-	β1*01, β1*15, β5	-	+	F
13373p	-	-	β1*03, β1*03, β3*01, β3*01	-	-	-
13378p	-	-	β1*1301, β1*15, β3*03, β5	-	+	F
13390p	-	-	β1*0401, β1*15, β4, β5	-	-/+	F
13415p	-	-	β1*07, β1*15, β4, β5	-	-/+	F
13451p	-	-	β1*01, β1*15, β5	-	-	-
14331p	-	-	β1*07, β1*15, β4, β5	-	+	D
14377p	-	-	β1*01, β1*0401, β4	-	-/+	F
11462r	+	β1*07	β1*16, β5	β4	++	FD
12433r	+	β3	β1*03, β1*1401	β3	-	-
13389r	+	β3*02	β1*1101, β1*15	β5	+	FD
10842r	+	-	β1*1103, β3	β1*09, β4[§]	+	FD
12951r	+	-	β1*01, β1*01[¶]	-	-/+	FD
10898r	-	-	β1*03, β1*07, β3*01, β4	-	+	D
11115r	-	-	β1*0401, β1*15, β4, β5	-	-	-
11273r	-	-	β1*0401, β1*07, β4, β4	-	-/+	F
11387r	-	-	β1*03, β1*16, β3*01, β5	-	-	-
11751r	-	-	β1*0401, β1*08, β4	-	-/+	F
11982r	-	-	β1*09, β1*15, β4, β5	-	++	D
12023r	-	-	β1*0401, β1*15,	-	nr	

Sample ID	HLA-DR	HLA-DRβ Allotype Expression by Tumor Cells			CD4+ TIL	Distribution
		Expressed	Not Expressed	Undetermined		
12987r	-	-	β4, β5 β1*07, β1*08, β4	-	+	FD
13149r	-	-	β1*09, β1*09, β4, β4	-	-	-
13283r	-	-	β1*03, β1*10, β3*01	-	-/+	F

[†]p = primary tumor; r = recurrent tumor ; CD4+ TIL scored as defined in methods; nr=no result anti-CD4 IHC was not performed on these breast carcinoma samples due to limited tissue availablity, F=focal aggregates of CD4+ TIL, D=diffusely distributed CD4+ TIL.

[‡]In the case of HLA-DRβ allotype homozygosity, if expression levels were comparable to infiltrating inflammatory cells both allotypes were considered expressed.

[§]Allotype is likely expressed due to differences in % of tumor cells positive for other alleles and % positive for total DR (L243+), however an allotype distinguishing mAb was not available to ascertain expression.

[‖]100% of tumor cells were HLA-DR+ by L243 mAb, however, allotype specific mAbs were not utilized due to lack of tissue availability.

[¶]Although ≥ 25% of tumor cells were positive for generic HLA-DR (L243+), individual allotype specific mAbs stained <25% of cells.

Supplementary Table 3S4: Association of tumor cell HLA-DRβ allotype expression with prognostic parameters in breast carcinoma patients.

HLA-DRβ expression[†]	Diagnosis Age (Yrs)			ER (fmol/mg)			PR (fmol/mg)			Tumor Size (cm)			LN+ (%)		
	N	Mean±SEM (Median)	*P*[‡]	N	Mean±SEM (Median)	*P*	N	Mean±SEM (Median)	*P*	N	Mean±SEM (Median)	*P*	N	%	*P*
HLA-DR-	63	61.7±1.8 (65.0)	-	63	49.8±8.8 (16.3)	-	63	74.0±21.7 (17.9)	-	60	3.3±0.3 (2.7)	-	63	49.2	-
DRβ1*04 expressed	17	59.2±2.4 (56.0)	**0.001**	17	28.2±10.3 (13.9)	**0.006**	17	45.3±16.6 (14.5)	0.063	17	2.8±0.6 (2.5)	0.053	17	64.7	0.420
DRβX expressed	19	48.4±3.1 (46.0)		19	7.7±2.5 (3.2)		19	24.8±9.0 (6.3)		19	3.6±0.4 (3.2)		18	61.1	
DRβ1*0401 expressed	10	60.6±3.8 (57.0)	**0.004**	10	17.0±4.2 (15.5)	**0.012**	10	32.2±9.8 (21.6)	**0.018**	10	2.1±0.3 (1.9)	**0.032**	10	50	0.270
DRβX expressed	26	50.8±2.5 (50.5)		26	17.5±7.1 (3.7)		26	35.3±12.3 (6.3)		26	3.7±0.5 (3.1)		25	68	
DRβ1*07 expressed	7	50.1±3.8 (53.0)	**0.018**	7	3.8±2.3 (1.2)	**0.006**	7	9.6±3.4 (6.3)	0.076	7	3.2±0.7 (3.6)	0.963	7	71.4	0.378
DRβX expressed	29	54.3±2.5 (52.0)		29	20.7±6.3 (9.1)		29	40.5±11.2 (13.0)		29	3.3±0.4 (2.7)		28	60.7	
DRB1*11 expressed	5	63.0±6.4 (60.0)	**0.006**	5	10.1±5.4 (3.8)	**0.041**	5	14.3±4.4 (11.1)	0.194	5	3.3±0.9 (3.0)	0.997	5	100	0.085
DRβX expressed	31	52.0±2.2		31	18.5±6.0		31	37.7±10.6		31	3.2±0.4		30	56.7	

HLA-DRβ expression[†]	Diagnosis Age (Yrs)			ER (fmol/mg)			PR (fmol/mg)			Tumor Size (cm)			LN+ (%)		
	N	Mean±SEM (Median)	$P^{‡}$	N	Mean±SEM (Median)	*P*	N	Mean±SEM (Median)	*P*	N	Mean±SEM (Median)	*P*	N	%	*P*
		(51.0)			(4.8)			(12.4)			(2.7)				
DRβ1*13 expressed	6	46.7±3.9 (47.5)	**0.008**	6	28.7±22.1 (4.5)	0.059	6	87.7±41.6 (45.0)	0.174	6	4.8±1.3 (3.3)	0.118	6	100	0.058
DRβX expressed	29	54.6±2.5 (53.0)		29	15.6±4.8 (4.8)		29	24.5±6.5 (12.4)		29	2.9±0.4 (2.5)		29	55	
DRβ3 expressed	7	52.7±4.3 (53.0)	**0.008**	7	29.7±18.4 (13.2)	**0.019**	7	82.3±36.8 (29.0)	0.118	7	4.0±1.3 (2.7)	0.892	7	85.7	0.185
DRβX expressed	27	52.3±2.4 (51.0)		27	14.2±5.2 (4.6)		27	23.5±6.5 (12.0)		27	3.0±0.3 (2.5)		26	53.8	
DRβ3*02 expressed	5	52.2±5.9 (53.0)	**0.007**	5	9.6±4.3 (4.1)	**0.034**	5	30.0±21.1 (4.6)	0.202	5	2.8±0.9 (2.6)	0.702	5	80	0.426
DRβX expressed	29	52.4±2.3 (51.0)		29	18.8±6.4 (4.8)		29	36.6±10.9 (12.4)		29	3.3±0.4 (2.8)		28	57.1	
Total	99	58.8±1.4 (60.0)	-	99	40.0±6.1 (13.1)	-	99	59.6±14.3	-	96	3.3±0.2 (2.7)	-	98	54.2	-

[†]Only those allotypes that were expressd in at least 10% of HLA-DR+ tumors are depicted; DRB4 was excluded as DRB4 expression could not be determined using the available panel of mAbs for a substantial proportion of tumors. HLA-DR+ tumors were stratified based on whether a particular HLA-DRβ allotype was expressed (e.g., DRβ1*04 expressed) or if other HLA-DRβ allotypes were expressed (DRβX expressed).

[‡]P-values calculated by comparison of HLA-DR- and both HLA-DRB allotype expression categories using one-way analysis of variance for diagnosis age, Pearson's chi-square analysis for LN status and Kruskal-Wallis H test for ER, PR and tumor size.

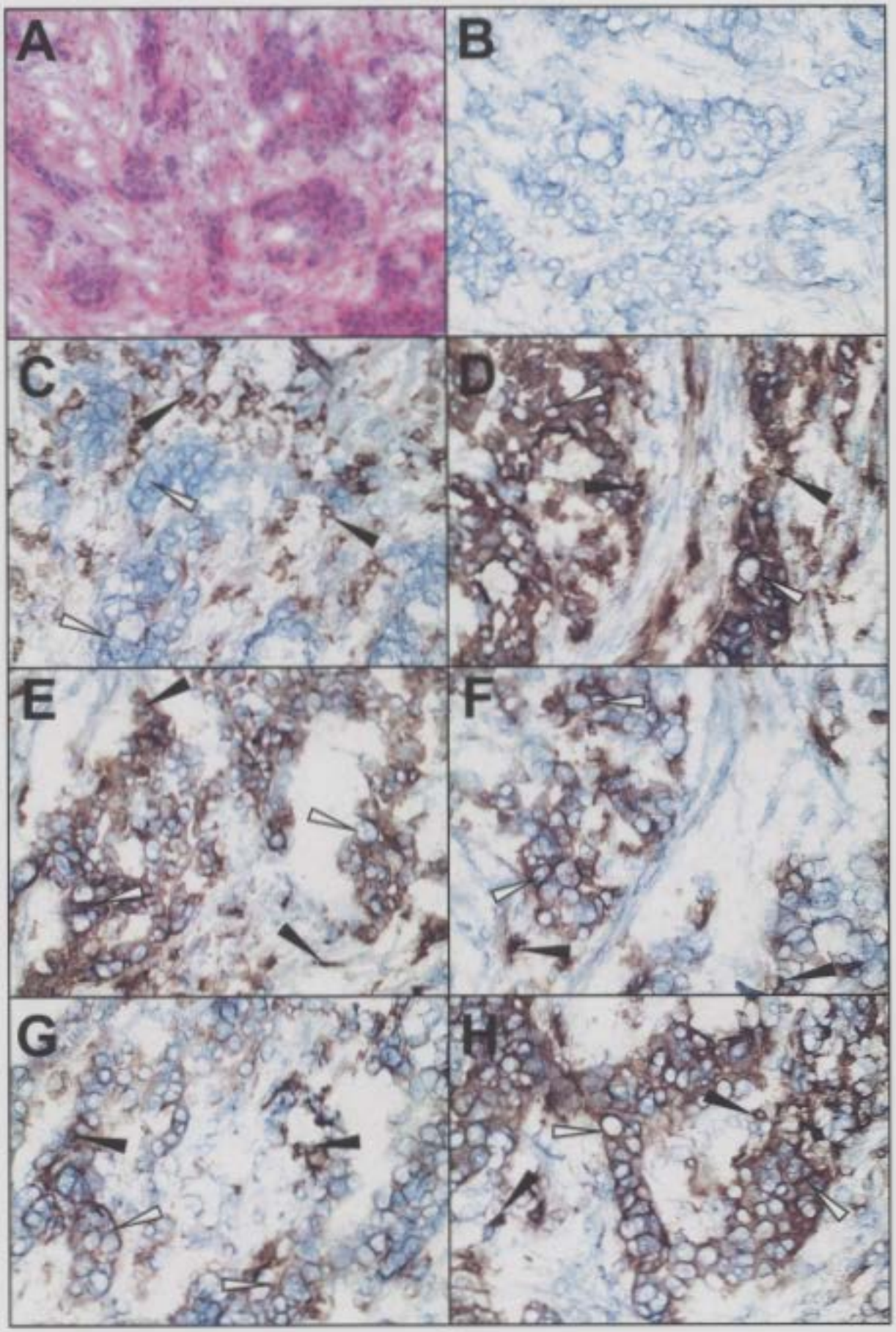
Supplementary Table 3S5: Multivariate Cox Proportional Hazards regression models using backward stepwise method[†] to test the effect of differential HLA-DRβ expression by breast tumor cells patient survival.

Variables remaining at last step	dRFS						DSS					
	Total			5 yr			Total			5 yr		
	p	HR	(95%CI)	P	HR	(95%CI)	p	HR	(95%CI)	p	HR	(95%CI)
HLA-DR expression	**0.005**	-	-	**0.006**	-	-	**0.003**	-	-	**0.004**	-	-
$DR\beta^{-/-}$ (N=56)		*Reference*			*Reference*			*Reference*			*Reference*	
$DR\beta^{-/+}$ (N=20)	**0.027**	2.42	(1.17-5.23)	**0.023**	2.50	(1.14-5.51)	**0.074**	2.07	(0.93-4.57)	**0.041**	2.34	(1.04-5.31)
$DR\beta^{+/+}$ (N=4)	0.976	0.00	-	0.977	0.00	-	0.981	0.00	-	0.983	0.00	-
$DR\beta^{UD}$ (N=11)	0.704	0.79	(0.23-2.74)	0.753	0.82	(0.23-2.87)	0.124	0.20	(0.03-1.55)	0.156	0.23	(0.03-1.76)
TNM Stage III/IV (vs I/II)	**0.002**	3.21	(1.53-6.77)	0.006	2.91	(1.36-6.25)	ns			ns		
PR<10fmol/mg	**0.019**	2.46	(1.16-5.23)	0.017	2.52	(1.18-5.38)	**0.004**	3.05	(1.42-6.56)	0.008	2.90	(1.31-6.41)
Model χ^2 (p-value)[‡]	24.0 (<0.001)			23.0 (<0.001)			19.8 (0.001)			19.3 (0.001)		
# events/# at risk	32/91			31/91			28/91			26/91		

[†]Cox proportional hazards models were analyzed using backward stepwise method with entry at $P<0.05$ and removal at $P\geq0.06$. All models controlled for treatment, tumor type, TNM stage, Diagnosis age, ER, and PR.

[‡] Model statistics

Supplementary Figure 3S1: Representative immunohistochemistry example of breast tumor cells that homogenously express HLA-DRβ allotypes. **(A)** Hematoxylin and eosin staining illustrating invasive ductal morphology. **(B-H)** Indirect immunohistochemistry using monoclonal antibodies (mAb) was performed on acetone-fixed breast carcinoma tissue sections. **(B)** Staining with IgG isotype control antibodies served as a negative control. **(C)** Infiltrating CD4+ (mAb RPA-T4) cells were detected in and around tumor nests. In this representative example (Tumor ID# 12450), total tumor DNA was HLA-DR typed as HLA-DRB1*0406, DRB1*1302, DRB3*03 and DRB4. **(D)** Tumor cells expressed generic HLA-DR (mAb L243) and expressed the **(E)** HLA-DRβ1*04 (mAb NFLD.D1), **(F)** HLA-DRβ1*13 (mAb UK8.1), **(G)** HLA-DRβ4 (mAb PL3) and **(H)** HLA-DRβ3*03 (mAb 7.3.19.1) allotypes. Infiltrating inflammatory and stromal cells served as positive controls for the immunoreactivity of mAb (closed arrowheads). Representative tumor cells are depicted by open arrowheads. Original magnifications of 200X (A) and 400X (B-H).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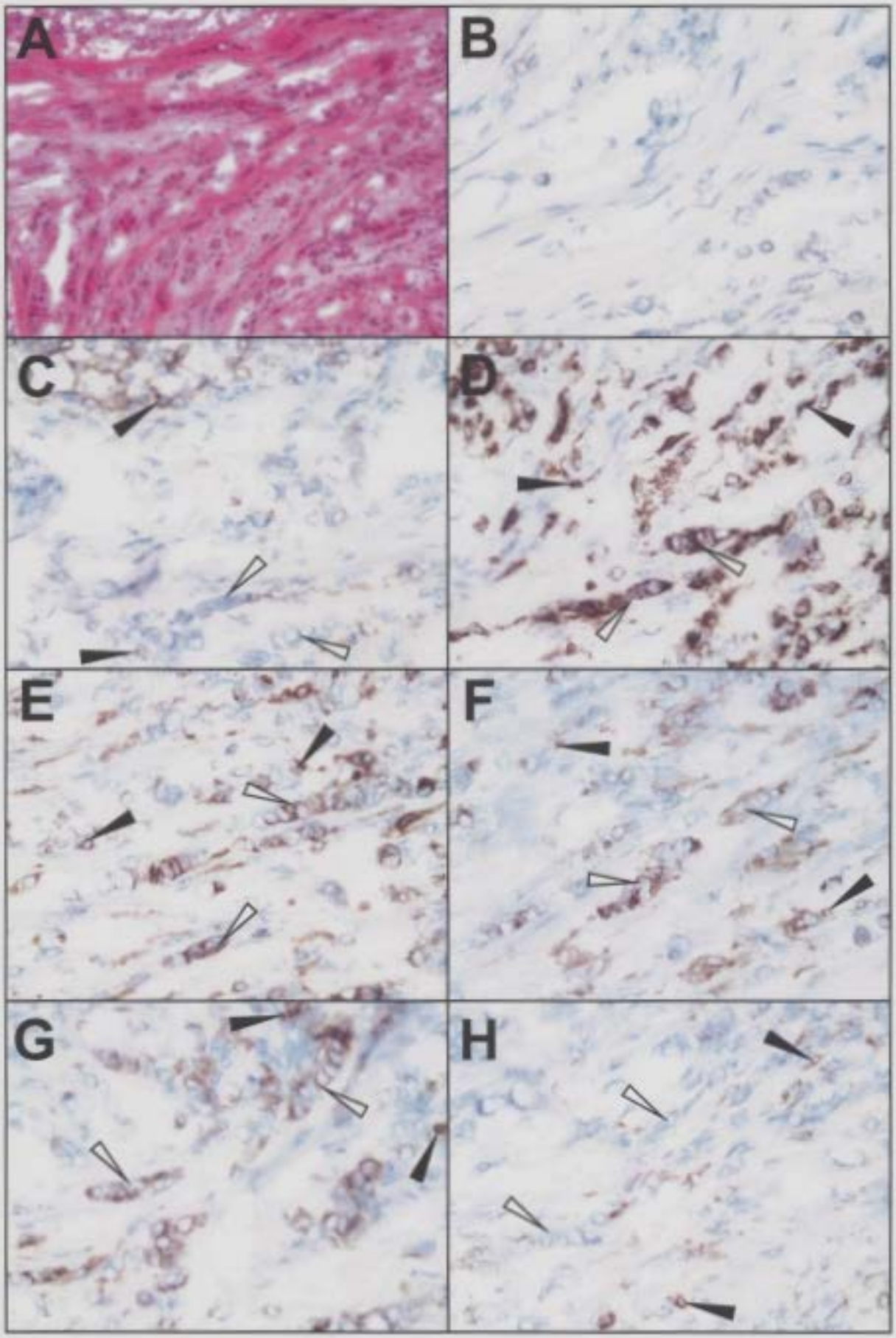
Supplementary Figure 3S2: Representative immunohistochemistry example of breast tumor cells that differentially express HLA-DRβ allotypes. **(A)** Hematoxylin and eosin staining illustrating invasive lobular morphology. **(B-H)** Indirect immunohistochemistry using monoclonal antibodies (mAb) was performed on acetone-fixed breast carcinoma tissue sections. **(B)** Staining with IgG isotype control antibodies served as a negative control. **(C)** Infiltrating CD4+ (mAb RPA-T4) cells were detected mainly as focal aggregates within the tumor. In this representative example (Tumor ID# 13091), total tumor DNA was HLA-DR typed as HLA-DRB1*0401, DRB1*1101, DRB3*0202 and DRB4. **(D)** Tumor cells expressed generic HLA-DR (mAb L243) and expressed the **(E)** HLA-DRβ1*04 (mAb NFLD.D1), **(F)** HLA-DRβ1*11 (mAb UK8.1) and **(G)** HLA-DRβ3*03 (mAb 7.3.19.1) allotypes but failed to express the **(H)** HLA-DRβ4 (mAb PL3) allotype. Infiltrating inflammatory and stromal cells served as positive controls for the immunoreactivity of mAb (closed arrowheads). Representative tumor cells are depicted by open arrowheads. Original magnifications of 200X (A) and 400X (B-H).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CHAPTER 4: TUMOR CELL EXPRESSION OF HLA-DM ASSOCIATES WITH A T_H1 PROFILE AND PREDICTS IMPROVED SURVIVAL IN BREAST CARCINOMA PATIENTS[‡]

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4.1 ABSTRACT

Studies aimed at elucidating the immunological and prognostic significance of HLA-DR expression on breast carcinoma cells have yielded contradictory results. To expand on previous studies, we have investigated the associations of tumor cell expression of HLA-DR and its related co-chaperones, invariant chain (Ii) and HLA-DM with infiltrating inflammatory cells, *in situ* cytokine mRNA levels and prognosis and outcome in 112 breast carcinoma patients with a median follow-up of 59 months. While the majority of HLA-DR+ tumors co-express Ii, only a minority express HLA-DM. Tumor cell expression of HLA-DR and co-chaperones positively associated with both infiltrating CD4+ and CD8+ T cell subsets ($P<0.01$). Expression of HLA-DR and Ii associated with decreased estrogen receptor alpha levels and younger age at diagnosis, suggesting a role for hormones in the control of HLA class II expression in breast carcinoma. Patients with DR+Ii+DM- tumors had markedly decreased recurrence-free and disease-specific survival as compared to patients with DR+Ii+DM+ tumors ($P<0.05$) and HLA-DR/co-chaperone expression was an independent predictor of survival by multivariate Cox regression analysis, controlling for standard prognostic indicators. Tumors that co-express HLA-DR, Ii and HLA-DM have increased levels of IFN-γ, IL-2 and IL-12 mRNA, suggesting improved survival of patients with DR+Ii+DM+ tumors may be attributable to T_H1 dominated immunity. We conclude that expression of determinants of the immune response by tumor cells may influence breast tumor progression and patient outcome.

4.2 INTRODUCTION

Successful anti-tumor immunity is dependent on CD4+ T cells, which recognize tumor peptides, presented by HLA class II antigens (HLA-DR, -DP, -DQ) [166, 167]. HLA class II antigen expression is generally restricted to professional antigen presenting cells (dendritic cells, macrophages, and B cells) and thymic epithelial cells, but HLA class II antigens are also expressed in a subset of benign and malignant breast tumors [146]. As HLA class II antigens are not normally present on resting breast epithelial cells, in the context of breast carcinoma HLA class II expression is likely dependent on the hormonal or cytokine milieu [134-136, 143, 230]. Several groups have demonstrated that HLA class II-positive tumor cells can induce an anti-tumor T cell response [138, 168, 170]. Thus, one would expect to see a clear association between the two factors within breast carcinoma lesions, however, immunohistochemical studies attempting to relate the two have yielded discrepant results [145, 147, 162, 177].

Similarly, the prognostic implications of HLA class II expression on breast carcinoma cells remain unclear as some studies have reported HLA-DR expression on breast tumor cells associates with the favorable prognostic indicators of well differentiated tumors [147, 148] and hormone receptor expression [159], while others found no such relationships [146, 152]. The impact of HLA-DR expression on relapse-free and disease-specific survival has also not been elucidated. Although HLA-DR expression associated with improved survival in a small subset of lymph node negative patients [155], most have reported no association [152, 156, 247].

These conflicting findings may be resolved by addressing other parameters of the anti-tumor immune response. The aforementioned studies investigated associations of

tumor cell HLA-DR expression with clinicopathological parameters, but did not address co-expression of the HLA class II co-chaperones invariant chain (Ii) and HLA-DM. Since Ii plays a critical role in HLA-DR trafficking [73], and HLA-DM facilitates peptide loading of HLA-DR molecules [66], discordant expression could have a detrimental effect on antigen-presentation of tumor peptides by HLA-DR+ tumor cells. To date, there are no published reports of HLA-DM expression in breast carcinoma and two studies that examined co-expression of Ii and HLA class II found a greater subset of breast carcinomas expressed Ii than HLA-DR, followed by HLA-DP and HLA-DQ [146, 151]. In other carcinomas, high Ii expression is associated with poor prognosis and disease progression [160, 161], suggesting that Ii interferes with CD4+ T-cell recognition of endogenously-derived tumor peptides. This is supported by *in vitro* experiments in which HLA class II-transfected tumor cells are unable to present tumor antigens to CD4+ T cells in the presence of Ii [89, 138].

Previously, we showed an association between HLA-DR and Ii expression on breast carcinoma cells with CD3+ T-cell infiltration [248]. The effects of these factors on prognosis and outcome in breast carcinoma are expected to depend on the subsets of infiltrating inflammatory cells and the in situ cytokine milieu, which in turn influence class II expression on tumor cells and their ability to act as surrogate antigen presenting cells. Thus, in this study, we focused on the interrelationships of tumor cell expression of HLA-DR, Ii and HLA-DM with infiltrating cell subsets and prototypical T_H1 (IFN-γ, IL-2, IL-12) and T_H2/T_H3 (IL-4, IL-10, TGF-β) cytokine mRNA levels. These factors have been further evaluated for their associations with clinicopathological parameters and survival in invasive breast carcinoma patients.

4.3 MATERIAL AND METHODS

4.3.1 Patient Sample

One-hundred and twelve primary breast carcinoma lesions were obtained from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada), with approval of the local Human Investigation Committees. Tumor type was known for 108 tumors, comprised mostly of infiltrating ductal (IDC, n=85), with 20 infiltrating lobular (ILC) and 3 mixed IDC+ILC. Tumors spanned a wide range of grade (grades 5–9), determined for 103 tumors, using the Nottingham grading system [6] and were classified as I (score 5, n=15), II (scores 6-7, n=51) and III (scores 8-9, n=37). Tumor size was available for 109 tumors, categorized as small (<2 cm, n=28) or large (>2 cm, n=81). Clinical lymph node (LN) status was available for all but one patient, and 56 were LN+. One-hundred and eight tumors were staged according to AJCC guidelines [8], of which 16 were Stage I, 71 were Stage II, 18 were Stage III and 3 were Stage IV. The age at diagnosis was available for all patients and ranged from 32 to 86 years (median 60 years; mean±SD = 59.3±14.5 years). Estrogen receptor (ER) and progesterone receptor (PR) levels were determined for all tumors by ligand binding assay and values ranged from 0 to 331 fmol/mg (median 13.3 fmol/mg; mean±SD = 35.4±57.7 fmol/mg) and 0 to 1088 fmol/mg (median 14.7 fmol/mg; mean±SD = 59.6±138.0 fmol/mg), respectively. Using a cutoff for negativity of less than 10fmol/mg, 48 were ER- and 73 were PR-. Her-2/neu expression was assessed in 89 tumors, as described below, and 19 (21.3%) tumors over-expressed Her-2/neu. The median follow-up time was 59 months (mean±SD = 57±30 months; range 2-127 months), during which 34 patients died of breast cancer. The median time to recurrence was 51

months (mean±SD = 49.2±32.1 months), during which 45 patients experienced recurrences (32 distant, 8 regional and 5 distant+regional).

Tumor samples were assessed by immunohistochemistry for HLA and co-chaperone expression and infiltrating cells and RT-PCR for cytokine mRNA levels. In total, 77 tumors were examined by immunohistochemistry and RT-PCR, 27 were assessed by IHC only and 8 had only cytokine information.

4.3.2 Monoclonal Antibodies

Monoclonal antibodies (mAbs) (spent supernatants) were used to detect generic HLA-class I (W6/32, 1:150, ATCC, Manassas, VA, USA) and HLA-DR (L243, 2.4μg/ml, ATCC). Commercially available mAb (BD Biosciences Pharmingen, Mississauga, ON, Canada) were used to detect Ii chain (clone LN2, 2.5μg/ml), HLA-DM (clone MaP.DM1, 2.5μg/ml), Her-2/neu (clone CB11, 1/100; clone N12, 2μg/ml, Neomarkers, Quebec, Canada) and infiltrating CD3+ (clone UCHT1, 2.5μg/ml, Pharmingen), CD4+ (clone RPA-T4, 0.625μg/ml, Pharmingen), CD8+ (clone HIT8a, 0.625μg/ml, Pharmingen), CD20+ (clone HI(FB1), 2.5μg/ml, Pharmingen), and CD68+ (clone EBM11, 2.15μg/ml, DakoCytomation, Mississauga, ON, Canada) cells. Tumor cells were identified using anti-cytokeratin mAb (clone AE1/AE3, 5μg/ml, DakoCytomation). Negative controls consisted of isotype matched non-specific mouse immunoglobulins (Southern Biotechnology Associates, Inc., Birmingham, AL, USA).

4.3.3 Immunohistochemistry

Serial frozen sections (8 μm) were fixed in acetone for 10 minutes at –20°C, shipped from the Manitoba Breast Tumor Bank and stored at –70°C until

immunohistochemical staining. After thawing, drying and rehydrating in phosphate buffered saline (PBS) (pH 7.4), sections were treated with 1.5% hydrogen peroxide in PBS for 30 minutes to remove endogenous peroxidases, and nonspecific binding was blocked with 15% goat serum in PBS for 1 hour. Sections were incubated for 1 hour with primary antibody followed by incubation for 30 minutes with goat anti-mouse DAKO EnVision horseradish peroxidase labeled polymer (DAKO Diagnostics Canada Inc., Mississauga, ON, Canada). Antibody binding was visualized by incubating with diaminobenzidine + hydrogen peroxide (Sigma, Oakville, ON, Canada) for 5 minutes. The reaction was stopped with water and sections were counterstained in Mayer's hematoxylin. Infiltrating mononuclear cells served as intrinsic positive controls for the immunoreactivity of the monoclonal antibodies. Hematoxylin and eosin staining was performed on one section for each breast carcinoma and samples where tumor cells comprised <10% of the section area were excluded from analysis.

4.3.4 Immunohistochemistry Interpretation

All slides were coded and independently examined by two readers, in the absence of information on prognostic parameters and HLA class II alleles. The percentage of tumor cells expressing HLA-DR, Ii and HLA class I was coded based on comparison to expression levels on inflammatory cells within the same tissue section: - (0-24% tumor cells positive); -/+ (25-49% tumor cells as strong as inflammatory cells or 25-74% tumor cells weaker than inflammatory cells); + (50-100% tumor cells as strong as inflammatory cells or 75-100% tumor cells weaker than inflammatory cells). Since DR and Ii are not normally expressed on breast epithelial cells, for categorical analysis samples with DR or Ii up regulated in at least 25% of tumor cells (Codes -/+ and +) were classified as

positive. As HLA-DM was expressed at much lower levels than HLA-DR or Ii on infiltrating inflammatory cells and tumor cells, we used a cutoff value of 10% for HLA-DM expression. For HLA class I, only those coded + were considered positive as samples coded – or -/+ were considered to have a substantial down regulation of HLA class I. Likewise, only the percentage of tumor cells strongly positive for HLA class I was used for continuous variable analysis while total percentage of tumor cells positive (weak or strong) was included for HLA-DR, Ii and HLA-DM.

Infiltrating CD3+, CD4+, and CD8+, CD20+, and CD68+ cells were coded based on examination of the entire section and estimation of relative numbers: - (no or a few scattered cells); -/+ (small numbers of scattered cells or occasional small aggregates); + (moderate numbers of scattered cells, numerous small aggregates or occasional large aggregates); ++ (large numbers of scattered cells or several large aggregates).

Tumor cell expression of Her-2/neu was determined in 89 tumors and coded as 0 (<10% with membrane staining), 1+ (≥ 10% with weak, incomplete membrane staining), 2+ (≥ 10% with weak to moderate complete membrane staining) or 3+ (≥ 10% with strong complete membrane staining). As some tumors coded 2+ by IHC will not show gene amplification by fluorescent in situ hybridization [249], only those tumors coded 3+ were considered clinically positive for statistical analysis.

4.3.5 RNA extraction, and semi quantitative RT-PCR

Total RNA was isolated from breast tumor tissues by homogenization using Trizol reagent (Gibco BRL, Rockville, MD), followed by treatment with DNA-free reagent (Ambion, Austin, TX) to remove any contaminating DNA. Reverse transcription was performed on 1μg RNA using the First Strand cDNA Synthesis Kit (Pharmacia

Biotech, Quebec, Canada). PCR was performed using a Biometra T Gradient thermocycler (Montreal Biotech Inc., Quebec, Canada) to amplify cDNA using primer pairs synthesized by Gibco BRL. Primer sequences and product sizes are described in Table 4.1 and included β-actin [251], IFN-γ, IL-2, IL-4, IL-10, IL-12 [252] and TGF-β1 [254]. All PCR reactions were performed in a volume of 50μL with 200μM dNTPs (Gibco BRL) and 1μL cDNA. PCR buffer contained 20mM Tris-HCl (pH 8.4) and 50mM KCl. Samples containing water instead of test cDNA were included as contamination controls and cDNA from the cell lines Jurkat E6-1 (IL-2, IL-4, IL-10, TGF-β), C10/MJ (IFN-γ) and YAR (IL-12) were used as positive controls. Primers were used at concentrations of 20pM for β-actin and 10pM for all others. $MgCl_2$ (Gibco BRL) concentration was 1.5mM for β-actin and IL-4 and 2mM for all other reactions. 0.2μL of Taq DNA polymerase (Gibco BRL) was used for β-actin reactions, all others used 0.25μL. Reaction mixtures were amplified for 35 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 55°C (IFN-γ and IL-2) or 65°C (all others), and extension at 72°C for 1 min, followed by a separate 5 min extension step at 72°C. The intensity of amplified products was semi-quantified and normalized as a percent of β-actin using the ChemiImager 4000 with Alphaease 4.0 Software (Alpha Innotech Corporation, San Leandro, CA).

Table 4.1: Primer sequences for detection of cytokine mRNA in breast carcinoma lesions.

	Sense Primer (5'-3')	Anti-sense Primer (5'-3')	Product Size (bp)	GenBank Accession Number*
β-Actin	ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG	CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC	840	NM_005159
IFN-γ	AGT TAT ATC TTG GCT TTT CA	ACC GAA TAA TTA GTC AGC TT	356	J00219
IL-2	ACT CAC CAG GAT GCT CAC AT	AGG TAA TCC ATC TGT TCA GA	269	HSU25676
IL-12	CCA AGA ACT TGC AGC TGA AG	TGG GTC TAT TCC GTT GTG TC	355	NM_002187
IL-4	CCT CTG TTC TTC CTG CTA GCA TGT GCC	CCA ACG TAC TCT GGT TGG CTT CCT TCA	371	M23442
IL-10	ATG CCC CAA GCT GAG AAC CAA GAC CCA	TCT CAA GGG GCT GGG TCA GCT ATC CCA	352	NM_000572
TGF-β	GCC CTG GAC ACC AAC TAT TGC	AGG CTC CAA ATG TAG GGG CAG G	165	X02812.1

* All primer sequences were validated using published GenBank mRNA sequences.

4.3.6 Statistical Analysis

Contingency tables were analyzed using Pearson's chi-square test or Fisher's exact test for 2x2 tables when at least one expected count was ≤ 5 (two-sided). Non-parametric Mann-Whitney (2 category variables) and Kruskal-Wallis (≥2 category variables) tests were used when assessing statistical significance of continuous variables, which were not normally distributed by Kolmogorov-Smirnov test. One-way analysis of variance and Fisher's least significant difference post hoc test were used to assess diagnosis age, which was normally distributed.

Survival estimates were calculated using Kaplan-Meier method with log-rank statistic. Ninety-five percent confidence intervals (95% CI) around 5-year percent survival estimates were calculated using the standard error (SE) of the cumulative survival probability (95%CI = cumulative survival probability ±1.96 x SE). Estimates were calculated as time to regional or distant metastasis for recurrence-free survival (RFS), time to distant metastasis for distant recurrence-free survival (dRFS) and time to death from breast cancer for disease specific survival (DSS). For DSS, patients who died of other causes were censored from analysis at time of death. Cox proportional hazards models were constructed for multivariate survival analyses using backward stepwise method. No patients were lost to follow-up and patients not experiencing the event were censored at the time of last follow-up or at 5 years for 5-year survival. For survival analysis, normalized cytokine units were stratified into high and low categories by comparing the 4^{th} quartile to the 1^{st}-3^{rd} quartiles. For multivariate analysis, correlation matrices were constructed to ensure lack of collinearity of covariates. All analysis was performed using SPSS Version 11.5 statistical software.

4.4 RESULTS

4.4.1 Tumor cells discordantly express HLA-DR and co-chaperones in breast carcinoma samples

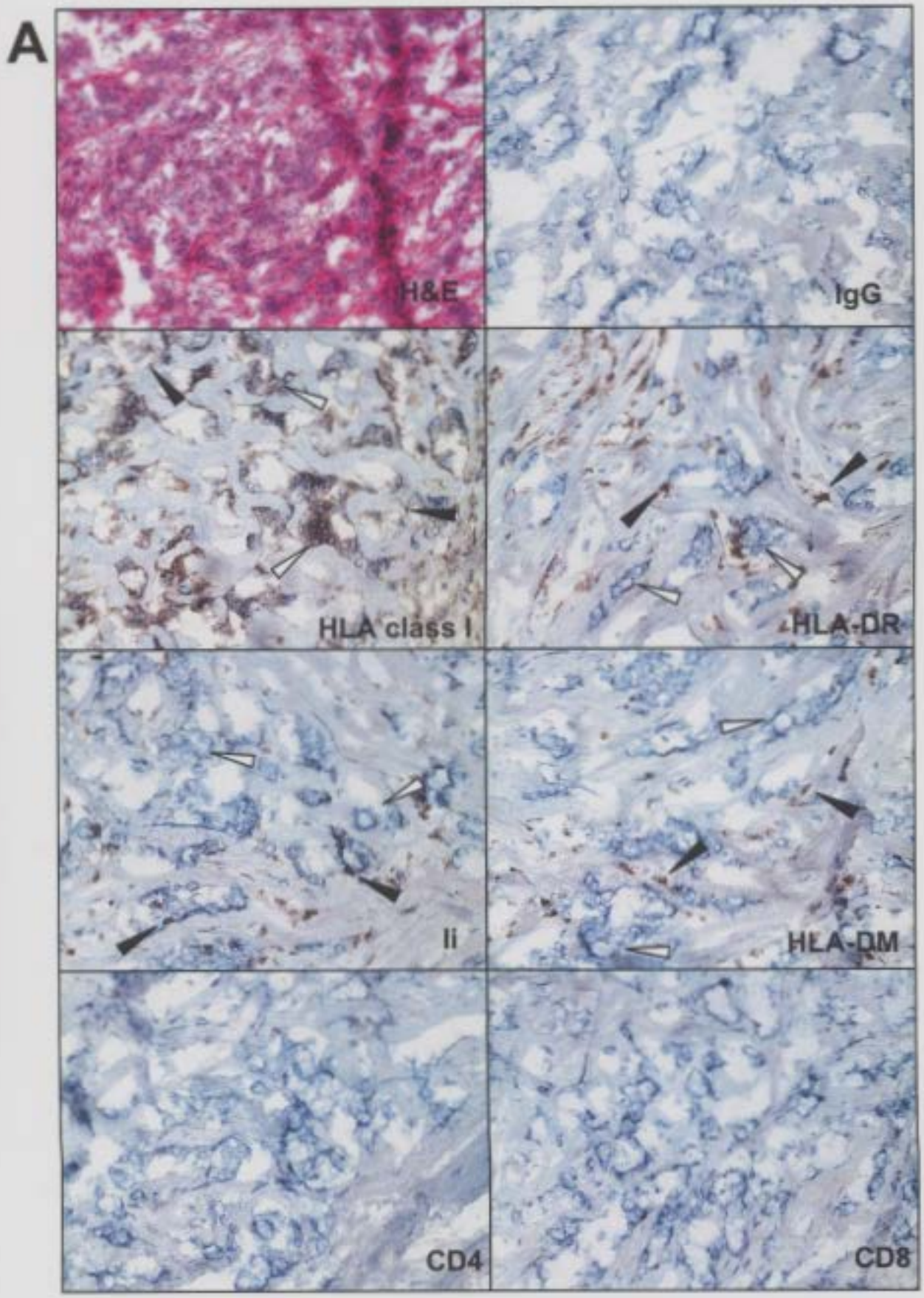
HLA-DR and Ii were assessed in 104/112 tumors and HLA-DM was assessed in 102/112. Following exclusion of samples containing <10% tumor cells, information on HLA-DR/Ii and HLA-DM expression was available for 99 and 97 tumors, respectively. Using categorical classification, there were 36 HLA-DR+ (mean ± standard deviation ($\bar{\times}_{\%}$) = 75±23.4%), 63 HLA-DR- ($\bar{\times}_{\%}$ = 1.3±4.1%), 51 Ii+ ($\bar{\times}_{\%}$ = 78.6±24.3%), 48 Ii-

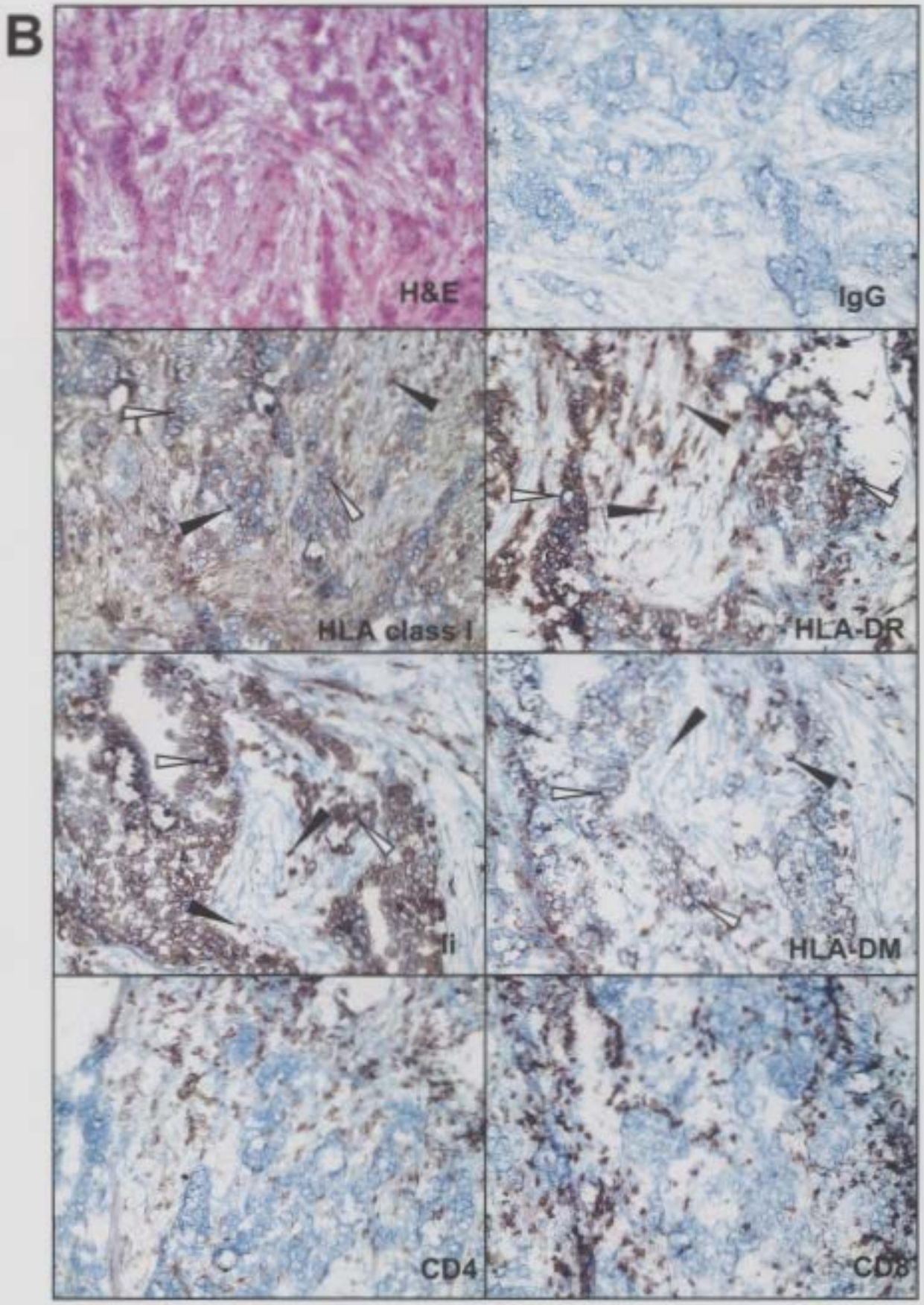
($\bar{x}_{\%}$ = 2±5.3%), 9 HLA-DM+ ($\bar{x}_{\%}$ = 28.9±14.5%), and 88 HLA-DM- ($\bar{x}_{\%}$ = 0±0%) tumors. Positive associations were observed between tumor cell expression of HLA-DR and Ii (χ^2=47.3, P<0.001), HLA-DM and HLA-DR (Fisher's exact test, P<0.001) and HLA-DM and Ii (Fisher's exact test, P=0.003) (not depicted). In total, 46 were DR-Ii-DM-, 9 co-expressed all three antigens and discordant expression was observed in 42 tumors with 25 DR+Ii+DM-, 1 DR+Ii-DM-, and 16 DR-Ii+DM-. Representative examples of breast tumor cells with the aforementioned HLA/co-chaperone phenotypes are depicted in Figures 4.1A-D. As an indirect measure of integrity of the HLA loci, tumor cell expression of generic HLA class I antigens was determined. Comparison of the percentage of tumor cells with strong HLA class I expression within HLA-DR/co-chaperone expression categories revealed decreased HLA class I in both HLA-DR- categories (Figure 4.2A), suggesting a negative effect on the HLA complex in HLA-DR- tumors.

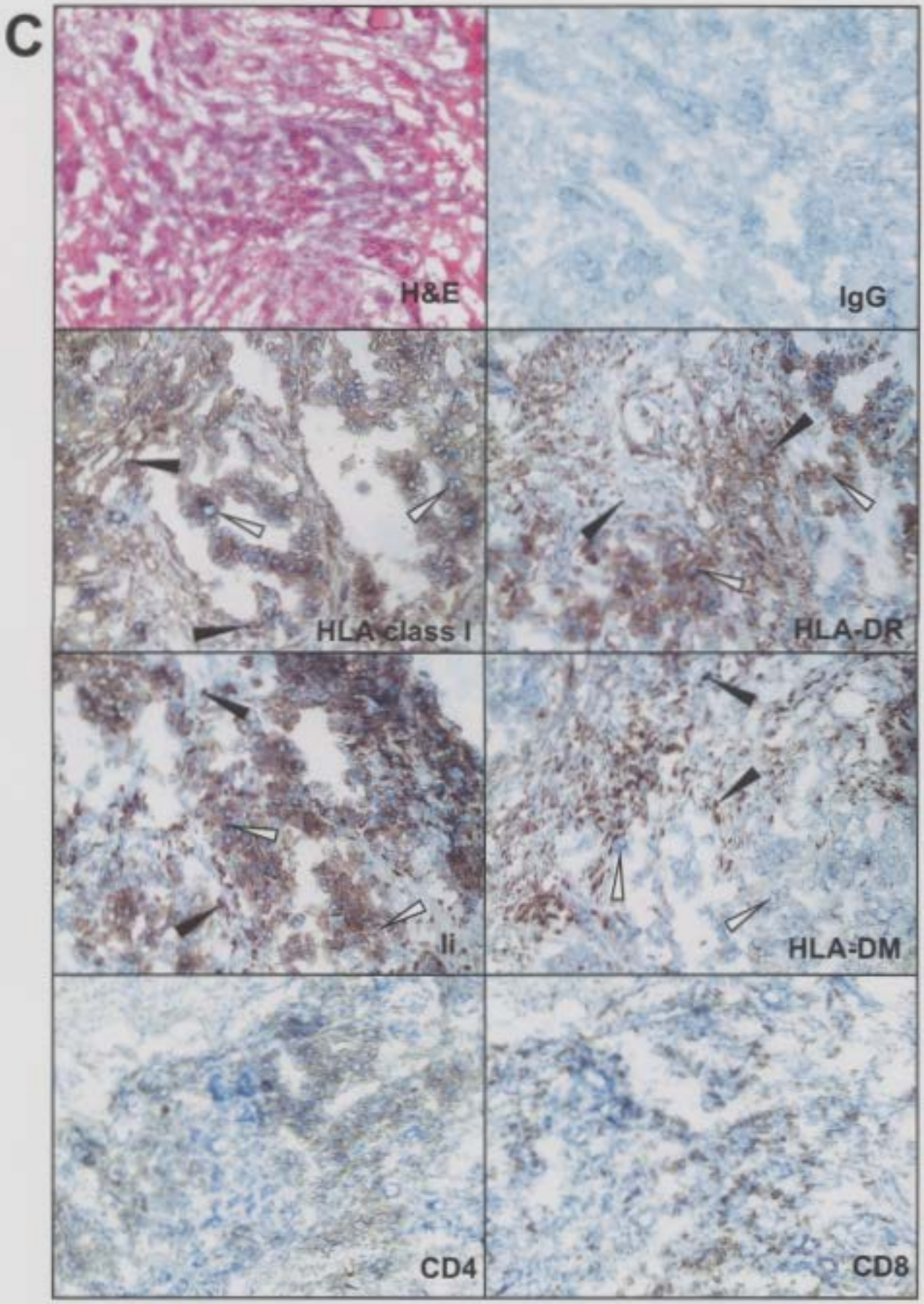
4.4.2 HLA-DR and co-chaperone expression associates with a TH1 cytokine profile in breast carcinomas

Tumor cell expression of HLA-DR, Ii and HLA-DM positively associated with infiltrating CD3+, CD4+ and CD8+ T cells (Figure 4.2B-D) but did not significantly differ with respect to infiltrating CD20+ and CD68+ cell numbers (Figure 4.2E-F). All tumors that co-express HLA-DR and co-chaperones contain infiltrating CD3+ T cells comprised of both CD4+ and CD8+ T cell subsets (Figure 4.3A and representative example depicted in Figure 4.1B). Tumor infiltrating T cell populations associate with both T_H1 and T_H2 cytokine mRNAs in invasive breast carcinoma (Oldford et al., manuscript in preparation, Chapter 5). Thus, relative amounts of T_H1 and T_H2/T_H3

Figure 4.1: Representative immunohistochemistry examples of breast tumor cell expression patterns of HLA-DR, Ii and HLA-DM and tumor infiltration by CD4+ and CD8+ TIL. Indirect immunohistochemistry using monoclonal antibodies (mAb) against HLA class I (mAb W6/32), HLA-DR (mAb L243), Ii (mAb LN2), HLA-DM (mAb MaP.DM1), CD4+ TIL (mAb RPA-T4) and CD8+ TIL (mAb HIT8a) was performed on acetone-fixed breast carcinoma tissue sections. Staining with IgG isotype control antibodies served as a negative control. Hematoxylin and eosin (H&E) staining illustrates invasive ductal morphology. Infiltrating inflammatory and stromal cells served as positive controls for the immunoreactivity of mAb. (closed arrowheads). Representative tumor cells are depicted by open arrowheads. **A)** Breast tumor cells lack expression of HLA-DR, Ii and HLA-DM and the tumor contains a paucity of CD4+ and CD8+ TIL. **B)** Breast tumor cells co-express HLA-DR, Ii and HLA-DM and CD4+ and CD8+ cells were detected in and around tumor nests. **C)** Breast tumor cells co-express HLA-DR and Ii but lack expression of HLA-DM and CD4+ and CD8+ cells were detected in and around tumor nests. **D)** Breast tumor cells express the co-chaperone Ii but fail to express HLA-DR and HLA-DM and CD4+ and CD8+ cells were detected in and around tumor nests. Original magnifications of 100X (H&E) and 200X (all others).


A
H&E
IgG
HLA class I
HLA-DR
Ii
HLA-DM
CD4
CD8


B
H&E
IgG
HLA class I
HLA-DR
Ii
HLA-DM
CD4
CD8


C
H&E
IgG
HLA class I
HLA-DR
Ii
HLA-DM
CD4
CD8

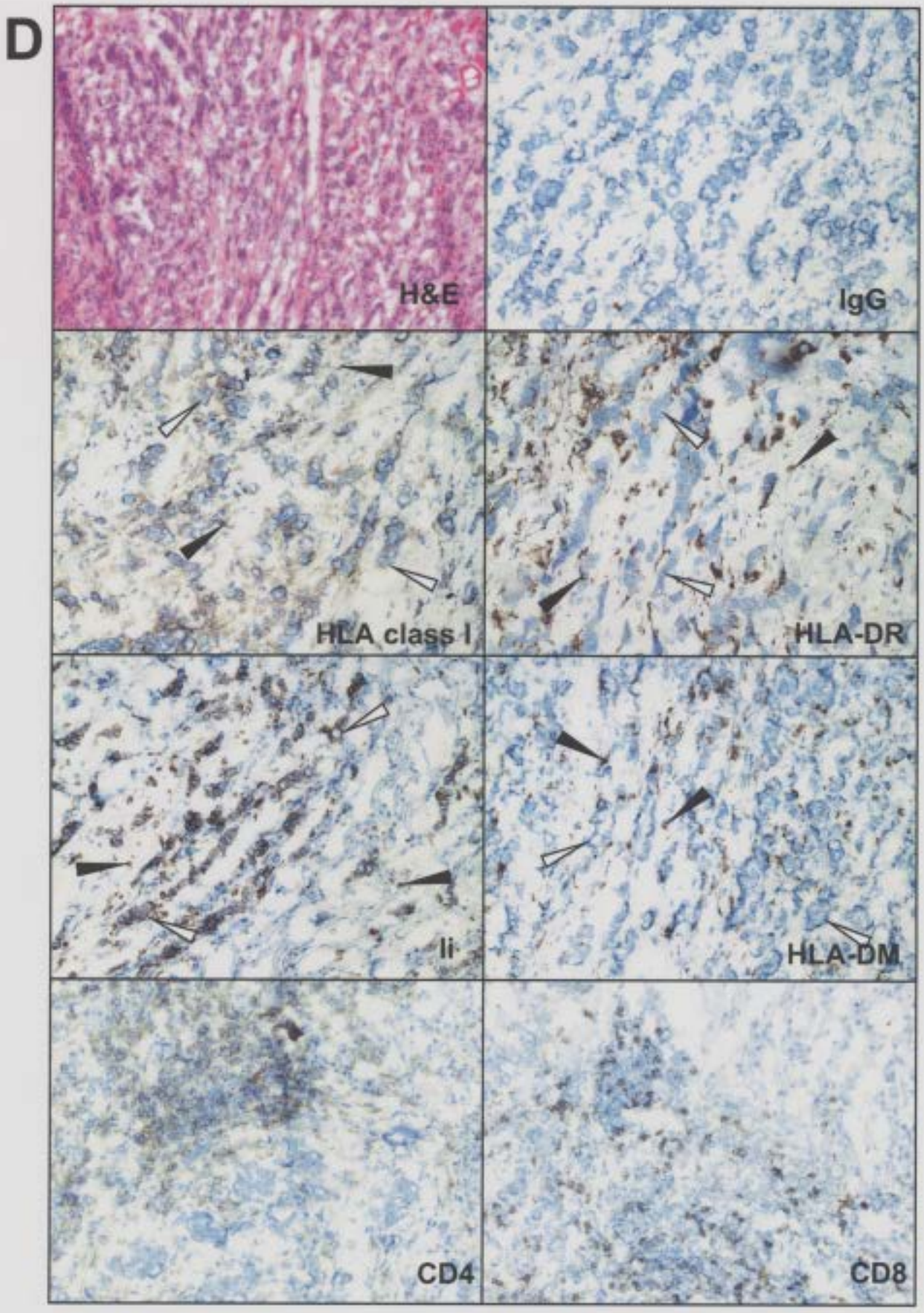
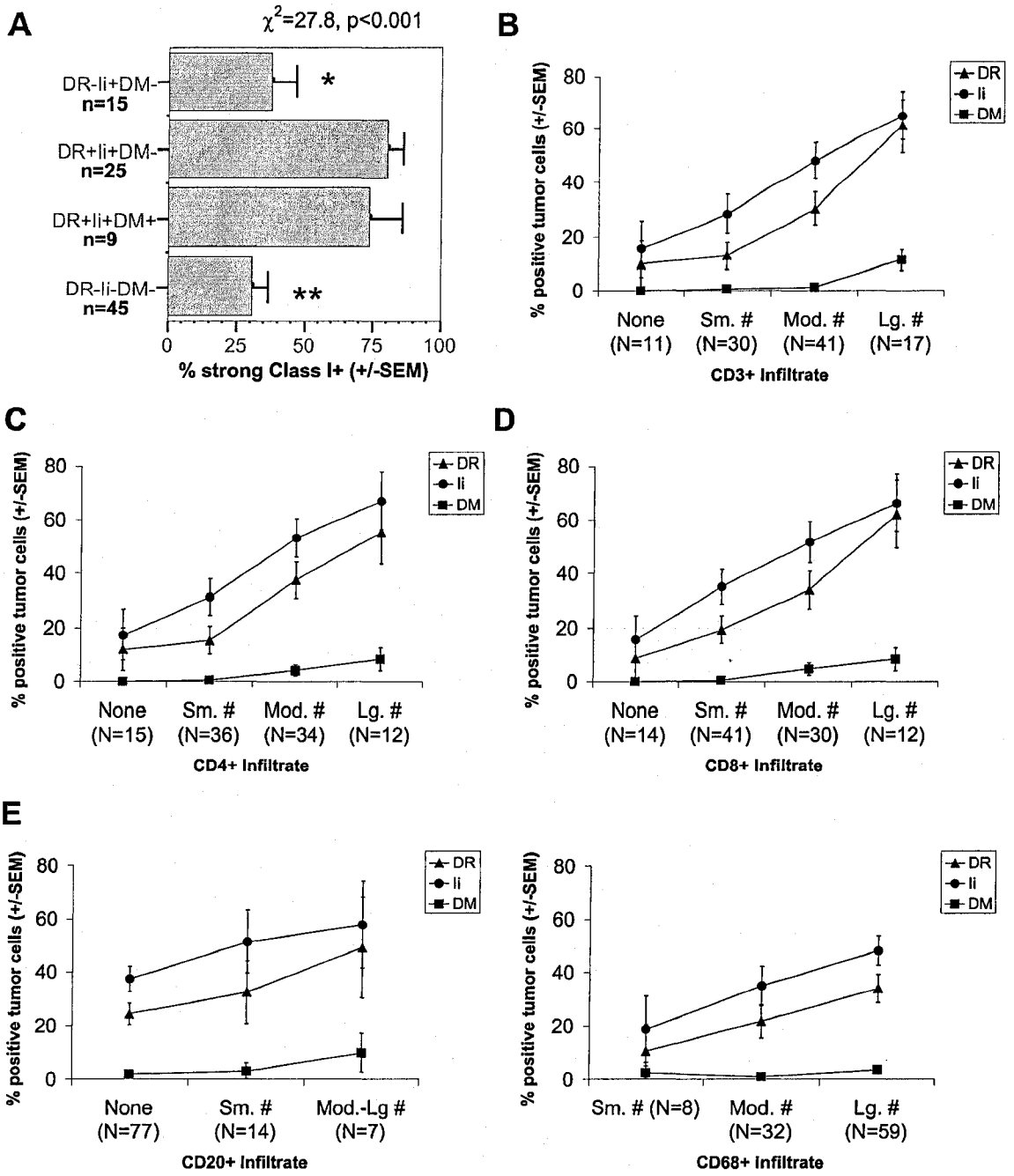

D
H&E
IgG
HLA class I
HLA-DR
Ii
HLA-DM
CD4
CD8

Figure 4.2: Associations of tumor cell HLA-DR and co-chaperones with HLA class I expression and infiltrating cells. **A)** HLA-class I expression is decreased in HLA-DR negative tumors. Chi-square and *P*-value in upper right corner corresponds to comparison of all groups using Kruskal-Wallis H test. Asterisks indicate significance in comparison to DR+Ii+DM+ tumors determined using Mann-Whitney U test (*$P<0.05$, ** $P<0.01$). **B-F)** Associations of infiltrating inflammatory cells with tumor cell expression of HLA-DR and co-chaperones in breast carcinoma were assessed using Kruskal-Wallis H test. **B)** Infiltrating CD3+ cells associated with tumor cell expression of HLA-DR ($P=0.0003$), Ii ($P=0.0008$) and HLA-DM ($P<0.0001$). **(C)** Infiltrating CD4+ cells associated with tumor cell expression of HLA-DR ($P=0.0012$), Ii ($P=0.0005$) and HLA-DM ($P=0.01$). **(D)** Infiltrating CD8+ cells associated with tumor cell expression of HLA-DR ($P=0.0024$), Ii ($P=0.0018$) and HLA-DM ($P=0.007$). **(E)** Infiltrating CD20+ did not significantly associate with tumor cell HLA-DR ($P=0.391$), Ii ($P=0.159$) or HLA-DM ($P=0.179$) expression. Only one tumor contained large numbers of CD20+ cells so moderate and large numbers were grouped for comparison. **(F)** Infiltrating CD68+ cells did not significantly associate with tumor cell HLA-DR ($P=0.168$), Ii ($P=0.056$) or HLA-DM ($P=0.384$) expression.


A
χ^2=27.8, p<0.001
DR-Ii+DM-
n=15
DR+Ii+DM-
n=25
DR+Ii+DM+
n=9
DR-Ii-DM-
n=45
*
**
0
25
50
75
100
% strong Class I+ (+/-SEM)
B
% positive tumor cells (+/-SEM)
DR
Ii
DM
None (N=11)
Sm. # (N=30)
Mod. # (N=41)
Lg. # (N=17)
CD3+ Infiltrate
C
None (N=15)
Sm. # (N=36)
Mod. # (N=34)
Lg. # (N=12)
CD4+ Infiltrate
D
None (N=14)
Sm. # (N=41)
Mod. # (N=30)
Lg. # (N=12)
CD8+ Infiltrate
E
None (N=77)
Sm. # (N=14)
Mod.-Lg # (N=7)
CD20+ Infiltrate
Sm. # (N=8)
Mod. # (N=32)
Lg. # (N=59)
CD68+ Infiltrate

cytokine mRNAs were compared in tumors stratified by HLA-DR/co-chaperone expression. All DR+Ii+DM+ tumors had detectable IFN-γ, IL-2 and IL-12 mRNA (Figure 4.3B) and levels of these T_H1 cytokines were significantly increased in DR+Ii+DM+ tumors as compared to tumors that lack expression of one or more molecules (Figure 4.3C). The prototypical T_H2/T_H3 cytokines, IL-4, IL-10 and TGF-β, did not significantly differ between expression categories (data not shown).

4.4.3 Associations of tumor cell HLA class II and co-chaperone expression with prognostic indicators in breast carcinoma patients

The clinical significance of tumor cell expression of HLA-DR and co-chaperones was assessed by examining associations with prognostic parameters. The age at diagnosis was decreased in patients with HLA-DR+ tumors, as compared HLA-DR- tumors (Figure 4.4A). Tumors that express HLA-DR and Ii in the absence of HLA-DM had significantly lower ER expression as compared to DR-Ii-DM- (Figure 4.4B). Likewise, ER-negative tumors had increased HLA-DR (ER-: $\bar{\times}_{\%}$ = 37.4±39.8% vs ER+: $\bar{\times}_{\%}$ = 20.9±36.1%; P=0.021) and Ii (ER-: $\bar{\times}_{\%}$ = 54.5±43.0% vs ER+: $\bar{\times}_{\%}$ = 31.4±39.4%; P=0.003) expression (not depicted). Expression categories did not significantly associate with PR, tumor grade, tumor stage, tumor size, LN status or Her-2/neu over-expression (Figure 4.4C-F and data not shown). Although HLA-DR/co-chaperone expression categories did not associate with tumor differentiation, the percentage of tumor cells expressing Ii positively associated with tumor grade (Grade I: $\bar{\times}_{\%}$ = 25.4±38.8%; Grade II: $\bar{\times}_{\%}$ = 39.7±42.2%; Grade III: $\bar{\times}_{\%}$ = 53.3±42.9%; Kruskal Wallis χ^2=6.6 P=0.036, not depicted).

Figure 4.3: Association of tumor cell HLA-DR/co-chaperone expression categories with infiltrating T cells and cytokines in invasive breast carcinoma. **(A)** Co-expression of HLA-DR, Ii and HLA-DM by tumor cells associates with increased numbers of CD3+ (χ^2=37.9, P<0.0001), CD4+ (χ^2=25.4, P=0.003), and CD8+ (χ^2=23.1, P=0.006) infiltrating cells. **(B)** All DR+Ii+DM+ breast tumors have detectable IFN-γ, IL-2 and IL-12 mRNA. RT-PCR was carried out using total RNA (1 μg) prepared from fresh-frozen breast carcinoma tissue. β-actin was amplified to confirm the integrity of the cDNA. Bands are representative of 1-3 RT-PCR reactions. Cell lines were used as positive controls for all RT-PCR reactions. **(C)** Tumors that co-express HLA-DR, Ii and HLA-DM have increased IFN-γ (χ^2=11.0, P=0.012), IL-2 (χ^2=7.4, P=0.060) and IL-12 (χ^2=9.1, P=0.028). Relative amounts of cytokine mRNA were normalized to β-actin and averaged for each breast carcinoma sample. Chi-square and P-value in upper right corner corresponds to comparison of all three groups using Kruskal-Wallis H test. Asterisks indicate significance in comparison to DR+Ii+DM+ tumors determined using Mann-Whitney U test (*P<0.05, **P<0.01).

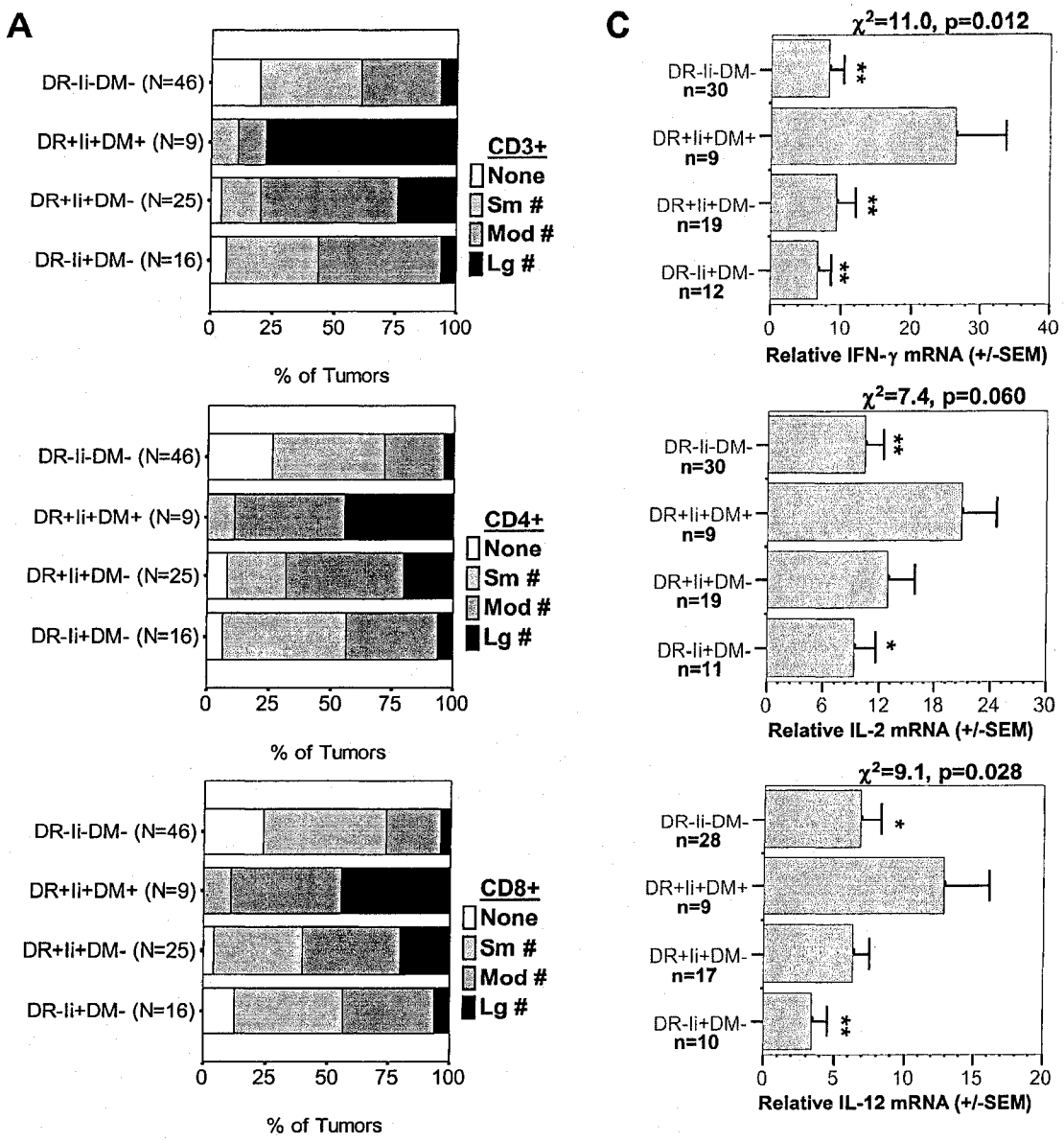
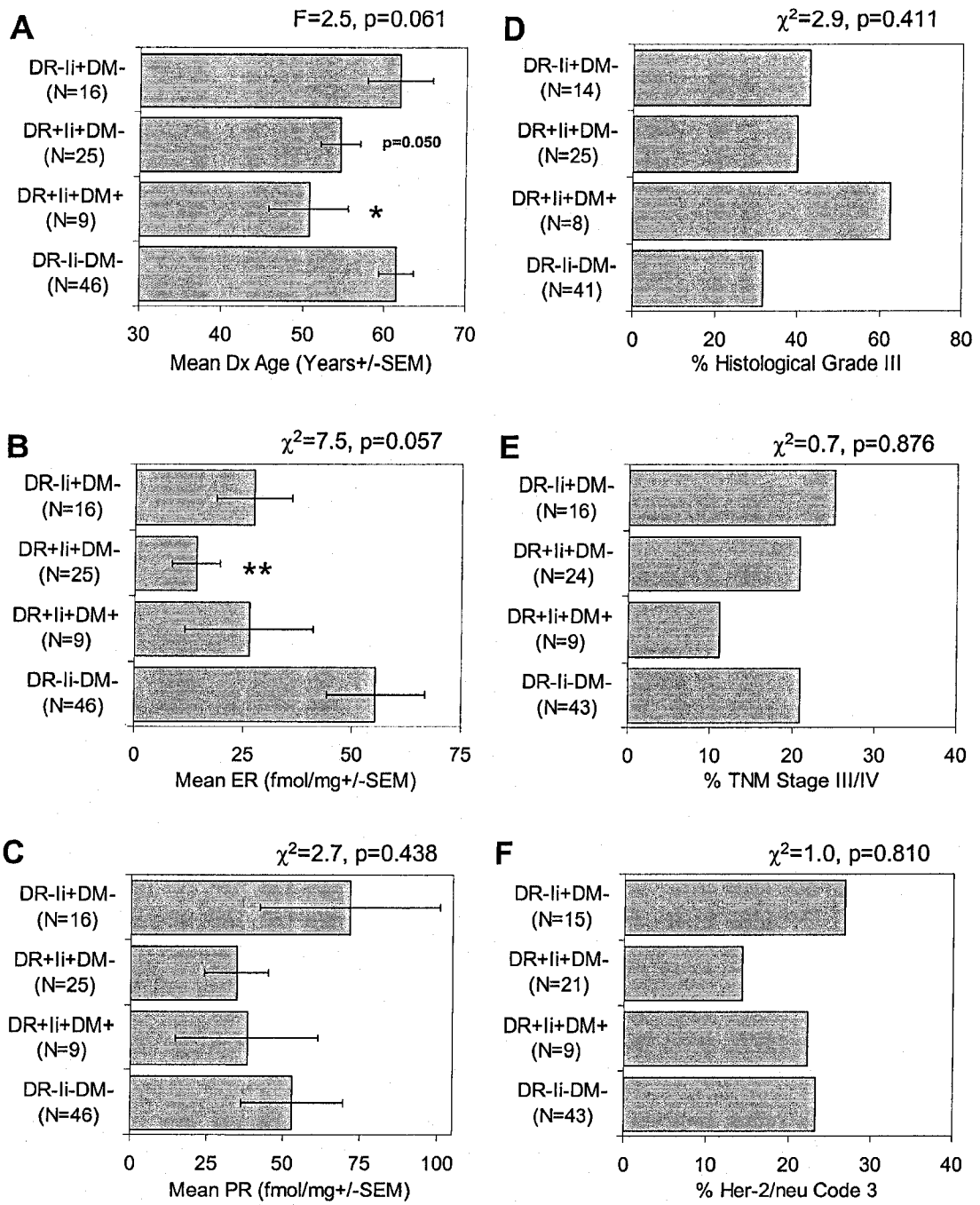

A
DR-Ii-DM- (N=46)
DR+Ii+DM+ (N=9)
DR+Ii+DM- (N=25)
DR-Ii+DM- (N=16)
CD3+
None
Sm #
Mod #
Lg #
0 25 50 75 100
% of Tumors
CD4+
CD8+
C
χ²=11.0, p=0.012
DR-Ii-DM- n=30
DR+Ii+DM+ n=9
DR+Ii+DM- n=19
DR-Ii+DM- n=12
0 10 20 30 40
Relative IFN-γ mRNA (+/-SEM)
χ²=7.4, p=0.060
DR-Ii-DM- n=30
DR+Ii+DM+ n=9
DR+Ii+DM- n=19
DR-Ii+DM- n=11
0 6 12 18 24 30
Relative IL-2 mRNA (+/-SEM)
χ²=9.1, p=0.028
DR-Ii-DM- n=28
DR+Ii+DM+ n=9
DR+Ii+DM- n=17
DR-Ii+DM- n=10
0 5 10 15 20
Relative IL-12 mRNA (+/-SEM)
B
DR+Ii+DM+ breast tumors
Control
10810
10970
11129
11452
12450
12483
13058
13123
13282
Jurkat
C10/MJ
YAR
IFN-γ
IL-2
β-actin
IL-12
β-actin

Figure 4.4: Association of tumor cell HLA-DR/co-chaperone expression categories with prognostic parameters. **A)** Diagnosis age is decreased in patients with DR+Ii+DM+ and DR+Ii+DM- tumors. **B)** DR+Ii+DM- tumors have significantly decreased ER levels as compared to DR-Ii-DM- tumors. Tumor cell HLA-DR/co-chaperone expression categories did not significantly associate with PR levels **(C)**, tumor differentiation **(D)**, tumor stage **(E)** or Her-2/neu over-expression **(F)**. F statistic or Chi-square and P-value in upper right corner correspond to comparison of all four groups using Kruskal-Wallis H test (χ^2) or one way analysis of variance (F-statistic). Asterisks and *P*-values indicate significance in comparison to DR-Ii-DM- tumors determined using Mann-Whitney U test for ER and Her-2/neu, and Fisher's least significant difference test for diagnosis age (*P<0.05, **P<0.01).

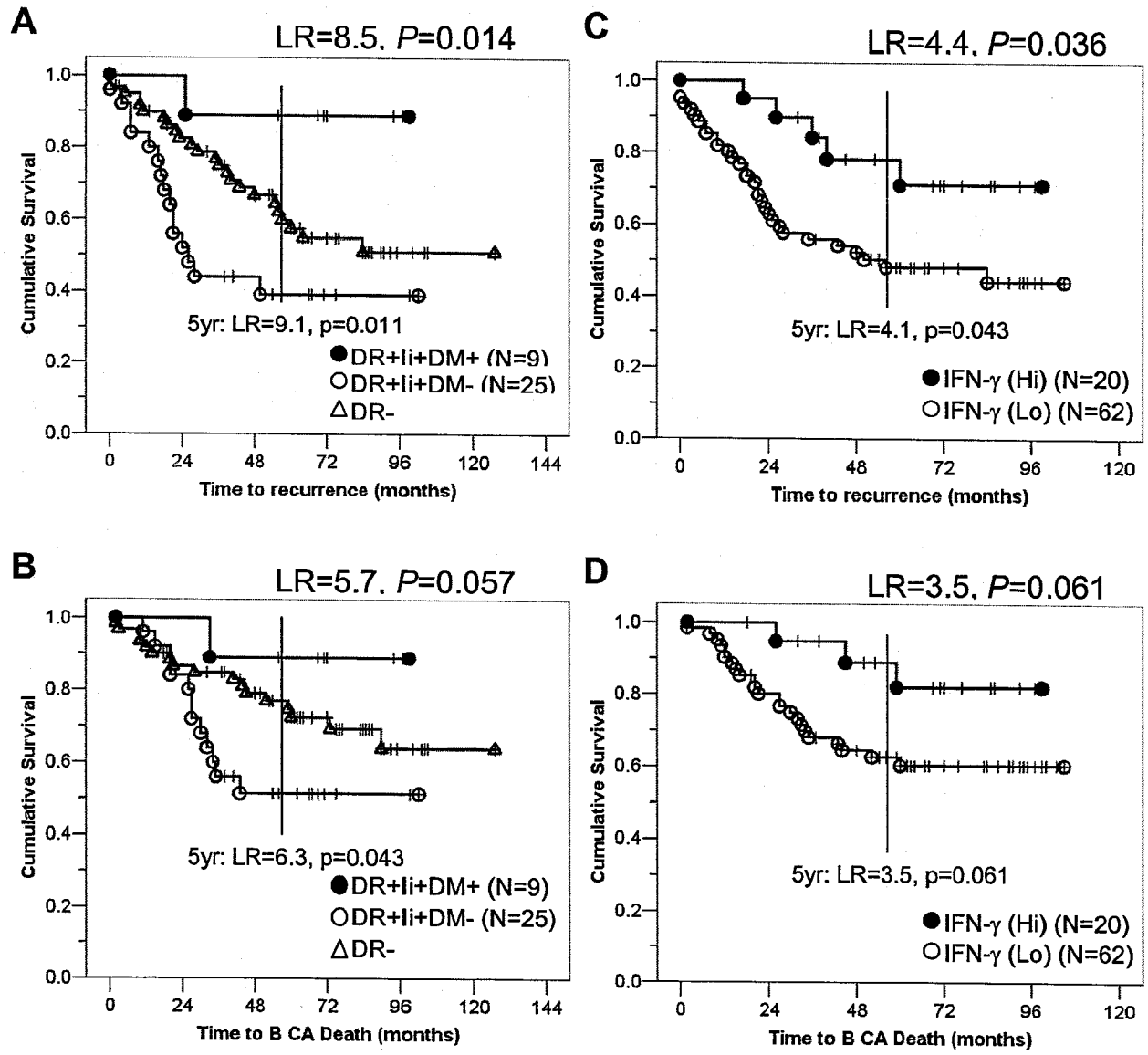

A
F=2.5, p=0.061
DR-Ii+DM-
(N=16)
DR+Ii+DM-
(N=25)
DR+Ii+DM+
(N=9)
DR-Ii-DM-
(N=46)
p=0.050
*
30
40
50
60
70
Mean Dx Age (Years+/-SEM)
D
χ²=2.9, p=0.411
DR-Ii+DM-
(N=14)
DR+Ii+DM-
(N=25)
DR+Ii+DM+
(N=8)
DR-Ii-DM-
(N=41)
0
20
40
60
80
% Histological Grade III
B
χ²=7.5, p=0.057
DR-Ii+DM-
(N=16)
DR+Ii+DM-
(N=25)
DR+Ii+DM+
(N=9)
DR-Ii-DM-
(N=46)
**
0
25
50
75
Mean ER (fmol/mg+/-SEM)
E
χ²=0.7, p=0.876
DR-Ii+DM-
(N=16)
DR+Ii+DM-
(N=24)
DR+Ii+DM+
(N=9)
DR-Ii-DM-
(N=43)
0
10
20
30
40
% TNM Stage III/IV
C
χ²=2.7, p=0.438
DR-Ii+DM-
(N=16)
DR+Ii+DM-
(N=25)
DR+Ii+DM+
(N=9)
DR-Ii-DM-
(N=46)
0
25
50
75
100
Mean PR (fmol/mg+/-SEM)
F
χ²=1.0, p=0.810
DR-Ii+DM-
(N=15)
DR+Ii+DM-
(N=21)
DR+Ii+DM+
(N=9)
DR-Ii-DM-
(N=43)
0
10
20
30
40
% Her-2/neu Code 3

4.4.4 Tumor cell co-expression of HLA-DR and HLA-DM predicts improved patient survival

Kaplan-Meier survival analysis was performed to assess whether expression of HLA-DR or HLA class II co-chaperones associated with recurrence-free survival (RFS), distant RFS (dRFS) or disease-specific survival (DSS). As there was no significant difference in survival between Ii+ or Ii- tumors that did not express HLA-DR (DR-Ii-DM- vs DR-Ii+DM-: LR=0.7, P=0.393 (RFS); LR=0.4, P=0.552 (dRFS); LR=0.5, P=0.461(DSS)), the two subsets were grouped for analysis. Patients with DR+DM+ tumors had prolonged RFS (Figure 4.5A), dRFS (Overall: LR=9.3, P=0.009; 5yr: LR=9.8, P=0.007, not depicted) and DSS (Figure 4.5B), while those with DR+DM- tumors had the shortest time to recurrence or breast cancer specific death. Infiltrating cell populations did not associate with breast carcinoma patient survival (data not shown). However, significant differences in patient survival were observed when tumors were stratified using quartile cut points for cytokine mRNA levels. Patients with tumors containing high amounts of IFN-γ mRNA had prolonged RFS (Figure 4.5C) and dRFS (Overall: LR=4.2, P=0.041; 5yr: LR=4.2, P=0.041, not depicted). Patients with high IFN-γ mRNA levels also had improved DSS although the difference was not statistically significant (Figure 4.5D). Patient survival did not differ in patients stratified by levels of IL-2, IL-12, IL-4, IL-10, or TGF-β mRNA (data not shown).

Cox proportional hazards models were constructed to determine if co-expression of HLA-DR and HLA-DM was an independent predictor of improved survival (Table 4.2). Cox regression models also contained treatment, tumor type, TNM stage, estrogen receptor, progesterone receptor, age at diagnosis and Her-2/neu status. Although

Figure 4.5: Association of tumor cell HLA-DR/co-chaperone expression and IFN-γ mRNA levels with patient survival. **A)** Patients with tumors that co-express HLA-DR, Ii and HLA-DM have improved recurrence-free survival as compared to patients with tumors that express HLA-DR and Ii in the absence of HLA-DM (5-yr % survival (95%CI) = 89% (68%-100%) for patients with DR+Ii+DM+ tumors, 39% (20%-59%) for patients with DR+Ii+DM- tumors and 58% (44%-71%) for patients with HLA-DR- tumors). **B)** Patients with tumors that co-express HLA-DR, Ii and HLA-DM have improved disease-specific survival as compared to patients with tumors that express HLA-DR and Ii in the absence of HLA-DM (5-yr % survival (95%CI) = 89% (68%-100%) for patients with DR+Ii+DM+ tumors, 51% (31%-71%) for patients with DR+Ii+DM- tumors and 72% (60%-84%) for patients with HLA-DR- tumors). **(C)** Patients with tumors containing high IFN-γ mRNA levels have improved RFS (5-yr % survival (95%CI) = 71% (49%-93%) for patients with high IFN-γ, 48% (35%-61%) for patients with low IFN-γ). **(D)** Patients with tumors containing high IFN-γ mRNA levels display a trend for increased DSS (5-yr % survival (95%CI) = 82% (63%-100%) for patients with high IFN-γ, 60% (48%-73%) for patients with low IFN-γ). Log rank (LR) statistic and P-value in upper right corners correspond to overall comparison, LR and P-value for 5-year survival rates are indicated on graph.


A
LR=8.5, P=0.014
Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
72
96
120
144
Time to recurrence (months)
5yr: LR=9.1, p=0.011
DR+Ii+DM+ (N=9)
DR+Ii+DM- (N=25)
ΔDR-
C
LR=4.4, P=0.036
Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
72
96
120
Time to recurrence (months)
5yr: LR=4.1, p=0.043
IFN-γ (Hi) (N=20)
IFN-γ (Lo) (N=62)
B
LR=5.7, P=0.057
Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
72
96
120
144
Time to B CA Death (months)
5yr: LR=6.3, p=0.043
DR+Ii+DM+ (N=9)
DR+Ii+DM- (N=25)
ΔDR-
D
LR=3.5, P=0.061
Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
72
96
120
Time to B CA Death (months)
5yr: LR=3.5, p=0.061
IFN-γ (Hi) (N=20)
IFN-γ (Lo) (N=62)

histological tumor grade significantly associated with tumor type, LN, ER, PR and diagnosis age, it was not associated with overall or 5-year RFS, dRFS or DSS (Appendix II). Therefore, grade was not included in the multivariate models as the dependence among the covariates made the parameter estimates indeterminate resulting in a lack of coefficient convergence. When controlling for the above prognostic factors, tumor cell HLA-DR/DM expression was an independent predictor of RFS (P=0.001), dRFS (P=0.001) and DSS (P=0.031). Tumor stage, PR and Her-2/neu status were also independent predictors for RFS and dRFS, while PR expression independently predicted DSS. Similar results were obtained for 5-year survival models (data not shown).

As fewer samples had information on cytokine mRNA, separate multivariate models were constructed to test the ability of IFN-γ to independently predict patient survival. When controlling for the aforementioned clinicopathological parameters, high IFN-γ displayed only a trend for improved RFS (at step 1: HR(95%CI)=0.38(0.12-1.21), P=0.101) and dRFS (at step 1: HR(95%CI)=0.33(0.09-1.23), P=0.099) and did not associate with DSS (at step 1: HR(95%CI)=0.32(0.06-1.74), P=0.188) (data not shown).

4.5 DISCUSSION

This study was aimed at investigating relationships of tumor cell expression of HLA-DR and the co-chaperones, Ii and HLA-DM with immune cell infiltration, the intratumoral cytokine profile and prognosis and outcome in breast carcinoma patients. HLA-DR, Ii and HLA-DM are typically coordinately regulated by the HLA class II transactivator (CIITA) [99], however, discordant expression by tumor cells was observed.

Table 4.2: Multivariate Cox Proportional Hazards regression models using backward stepwise method

Variables remaining at last step[†]	RFS		dRFS		DSS	
	P	HR (95%CI)[‡]	*P*	HR (95% CI)	*P*	HR (95%CI)
PR <10 fmol/mg (vs ≥10fmol/mg)	**0.037**	2.29 (1.05-4.97)	**0.025**	2.63 (1.13-6.15)	**0.003**	3.86 (1.57-9.51)
TNM Stage III/IV (vs Stage I/II)	**0.005**	3.03 (1.41-6.52)	**0.013**	3.02 (1.26-7.23)	0.070	2.47 (0.93-6.53)
Her-2/neu Code 3 (vs Codes 0-2)	**0.017**	2.49 (1.18-5.25)	**0.020**	2.71 (1.17-6.25)	ns	-
DR/DM category	**0.001**	-	**0.001**	-	**0.031**	-
DR^+DM^-	*Reference*		*Reference*		*Reference*	
DR^+DM^+	**0.010**	0.07 (0.01-0.52)	**0.014**	0.07 (0.01-0.58)	**0.045**	0.12 (0.01-0.95)
DR^-	**0.010**	0.37 (0.18-0.79)	**0.005**	0.31 (0.14-0.69)	0.143	0.53 (0.23-1.24)
Model χ^2 at last step (*P*-value)	22.6 (<0.001)		21.8 (0.001)		15.8 (0.003)	
# events/# at risk	35/82		28/82		25/82	

[†] All regression models controlled for treatment, tumor type, ER, PR, diagnosis age, TNM stage and Her-2/neu expression status.

[‡] Hazards ratio (95% confidence interval for hazards ratio)

Within HLA-DR+ tumors, 97% co-expressed Ii but only 26% were HLA-DM+. The underlying mechanisms responsible for discordant expression of HLA-DR and co-chaperones are currently unclear. Ii expression in the absence of HLA-DR and HLA-DM is likely attributable to CIITA independent transcription of Ii, owing to unique *cis* acting elements found within the Ii promoter [113]. Thus, *trans*-acting factors produced during tumorigenesis may facilitate Ii expression, possibly explaining the positive association of tumor cell Ii expression with increased histological grade of differentiation. Tumor cell expression of HLA-DM did not occur independently of HLA-DR or Ii and was observed only in those tumors with high levels of the T_H1 cytokines IFN-γ, IL-2, and IL-12. Discordant HLA-DM expression in HLA-DR+ tumors may be due to regulatory defects in HLA-DM since its promoters contain additional *cis* acting elements not shared by HLA class II or Ii promoters [112]. As such, additional *trans*-acting regulatory factors may be necessary for HLA-DM transcription. In support of this, the kinetics of HLA-DM induction on synovial fibroblast lines is much slower than that of HLA-DR or Ii, in response to IFN-γ treatment [267].

Discordant HLA-DM expression in the majority of HLA-DR+ tumors may also reflect a tumor escape mechanism similar to that reported for regulatory defects in HLA class I antigen processing machinery in breast carcinoma cell lines (BCCL). Down-regulated immunoproteasome subunits LMP-2, LMP-7, LMP-10 and transporter associated with antigen processing (TAP)-1 and TAP-2 molecules were documented in BCCL, and these defects could be restored via IFN-γ treatment [229]. Thus, if regulatory mutations are present and cause negative HLA-DM expression, they may be overcome in

the presence of high levels of intratumoral IFN-γ. In support of this all DR+Ii+DM+ tumors had detectable IFN-γ and relative levels were significantly higher than in tumors that lack expression of HLA-DM. We have observed, high dose IFN-γ treatment (500U/ml for 96hr) up-regulates HLA-DM protein expression in a majority of breast carcinoma cell lines (7/11 tested), and HLA-DM induction by IFN-γ is clearly dose-dependent (Oldford, Edgecombe and Drover, unpublished observations).

The negative association of HLA-DR and Ii expression with ER suggests that ER may negatively modulate HLA class II expression in breast carcinoma. Furthermore, the decreased age at diagnosis of patients with HLA class II expressing tumors implies a role for circulating estradiol levels. This finding was intriguing as 17β-estradiol is known to negatively modulate HLA-DR expression. In particular, 17-β estradiol inhibits IL-1α and IL-1β induction of HLA-DR in ER+ human endometrial and breast carcinoma cell lines in a dose dependent manner [135] and 17-β estradiol can down-modulate constitutive and IFN-γ induced MHC class II expression in a variety of murine and human cell types [262]. Its effects are independent of CIITA and involve ER binding to MHC class II promoters and activation of the c-Jun N-terminal kinase (JNK) pathway, leading to histone hypoacetylation and decreased association of CREB-binding protein (CBP) with the class II promoter [263]. Based on our findings we suggest that the down-modulatory effect of estradiol on HLA-DR expression may be overcome in ER- breast carcinoma cells.

The association of tumor cell expression of Ii with a poor prognosis in this study and in other types of carcinoma [160, 161], is hypothesized to be due to inefficient presentation of endogenous tumor antigen derived peptides by HLA class II molecules. It

is plausible that induction of HLA-DM expression by tumor cells allows for efficient exchange of Ii for endogenous tumor antigen derived peptides that have transected the endocytic pathway. HLA-DM is important for the exchange of the class II associated Ii peptide (CLIP) for exogenous antigenic peptides [66, 82] and high levels of HLA-DR/CLIP complexes are expressed by mutant B cells deficient in HLA-DM [81]. Similarly, we have observed increased surface HLA-DR/CLIP expression in a HLA-DM deficient breast carcinoma cell line as compared to HLA-DM expressing breast carcinoma cell lines (Oldford, Edgecombe and Drover, unpublished observations). Thus, improved survival of patients with DR+Ii+DM+ tumors over patients with DR+Ii+DM- tumors may reflect differences in antigen presentation and the resulting T cell response. Indeed, experimental studies showed high levels of CLIP, whether exogenously added or endogenously expressed by antigen presenting cells (APC), modulated antigen-specific effector T cells, inducing a shift from T_H1 to T_H2 responses [125, 126]. Interestingly, other self-peptides did not have a polarizing effect on the effector CD4+ T_H cell response.

Meazza et al. (2003), showed CIITA transfected TS/A mammary adenocarcinoma cells strongly up-regulated MHC class II antigens and Ii and were rejected in syngeneic mice in a CD4+ T cell dependent manner [168]. Recently, Thompson et al. (2006) demonstrated human breast carcinoma cells transduced with CIITA are able to present endogenous Her-2/neu peptides and activate human CD4+ T cells to secrete high levels of IFN-γ, with or without siRNA suppression of Ii [268]. Although not investigated in either study, the improved immunogenicity of these tumor cells may be due to up regulation of DM, which is also transcriptionally controlled by CIITA [99], thereby allowing for efficient exchange of CLIP for endogenous tumor antigen peptides. Indeed,

high levels of CLIP on myeloid leukemia blast cells predicted poor patient survival and associated with decreased HLA-DM expression [269].

We can only speculate about the mechanism for HLA-DM expression association with improved patient survival. Possibly this association represents a bystander effect of high IFN-γ produced by T_H1 CD4+, CD8+ and/or NKT cells activated by tumor infiltrating antigen presenting cells (APC). Once HLA-DR and HLA-DM are up-regulated on breast tumor cells, they may serve as efficient APC, driving the activation and cytokine production of T_H1 CD4+ T cells, resulting in improved anti-tumor immunity and improved patient survival. In support of this, high levels of IFN-γ associated with decreased time to recurrence by univariate analysis (Figure 4.4). Relative IFN-γ mRNA levels did not independently predict patient survival in this study, possibly owing to the small sample size, but IFN-γ is an independent predictor of survival in other solid tumors [270].

The intermediate survival of patients with HLA-DR- tumors may in part reflect the increased loss of HLA class I in HLA-DR- tumors (Figure 4.2). In a large study of 439 breast carcinoma lesions, total loss of HLA class I independently predicted improved patient survival [271], although not investigated, this may reflect enhanced NK cell recognition or increased susceptibility to apoptosis [247]. Decreased HLA class I expression in DR-Ii-DM- and DR-Ii+DM- tumors suggests a negative effect on chromosome 6p. Loss of heterozygosity (LOH) of chromosome 6p is likely to play a major role in the lack of HLA class II antigen expression, since this mechanism causes HLA class I haplotype loss in a high percentage of tumors and is frequently attributable to loss of an entire chromosome, as indicated by LOH at both 6p and 6q markers [238].

This coupled with epigenetic mutations such as DNA methylation or histone deacetylation [229] likely explains the total loss of HLA observed in these tumors.

The results of this study suggest that coordinate expression of HLA-DR, Ii and HLA-DM by tumor cells is an indicator of improved prognosis in breast carcinoma. Furthermore, tumor cells that coordinately express HLA-DR and components of the HLA class II antigen processing machinery may function as effective antigen presenting cells, facilitating the induction of effective T_H1 anti-tumor immunity. Investigations such as this provide improved understanding of the immune response to carcinomas and suggest targeting antigen processing aberrations may aid in the successful generation of specific immunotherapeutic approaches to cancer treatment.

CHAPTER 5: CHARACTERIZATION OF THE *IN SITU* IMMUNE RESPONSE IN INVASIVE BREAST CARCINOMA[†]

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5.1 ABSTRACT

Breast carcinomas are frequently infiltrated by tumor infiltrating lymphocytes (TIL), which constitute the major source of immune-modulating cytokines. However, the prognostic significance of this infiltration remains unclear and is likely dependent on the balance of immunoregulatory and pro-inflammatory cytokines. To obtain a comprehensive evaluation of the *in situ* immune response we utilized immunohistochemistry to characterize the pattern of inflammatory cell infiltration and RT-PCR to determine the relative levels of intratumoral cytokine mRNA levels. As an indirect measure of immunoregulatory TIL, the T_{reg} specific marker FOX-P3 was also assessed. The presence of TIL associated with decreased hormone receptor levels and earlier age at diagnosis, suggesting a role for circulating hormones in their recruitment and/or expansion. Invasive breast tumors with large TIL numbers contained elevated levels of T_H1, T_H2, pro-inflammatory and immunoregulatory cytokines, demonstrating the existence of an integrated immune response within the tumor microenvironment. Patients with minor CD8+ TIL infiltration, exhibited low level T_H1 cytokine production, elevated FOX-P3 mRNA and had decreased time to distant recurrence. In contrast, patients with large numbers of TIL had improved survival and demonstrated elevated levels of T_H1-type cytokines, which associated with decreased tumor diameter. Elevated TNF-α independently predicted patient survival, strengthened by an elevated TNF-α:TGF-β1 ratio, demonstrating the prognostic significance of intratumoral TIL is dependent on the balance of pro-inflammatory and immunoregulatory cytokines. Future studies aimed at understanding the mechanisms of regulation of the anti-tumor immune response will aid in the development of immunotherapeutic regimes in breast carcinoma.

5.2 *INTRODUCTION*

The majority of breast tumors contain an inflammatory infiltrate comprised mainly of macrophages and CD4+ and CD8+ T cells, with smaller numbers of B cells and a relative paucity of natural killer (NK) cells [174, 175]. Large numbers of CD68+ tumor associated macrophages (TAM) are generally associated with poor prognostic indicators such as high tumor grade and decreased hormone receptor expression [272, 273]; however the prognostic significance of tumor infiltrating lymphocytes (TIL) in breast carcinoma is presently unclear. Several studies have failed to demonstrate an association of TIL with patient survival [187, 191, 192] and contradictory reports have been published with respect to associations of TIL with lymph node (LN) metastasis [154, 186], tumor stage [181, 185], histological grade [162, 181], and hormone receptor expression [181, 186].

Although discrepant reports on associations of immune cell infiltration in breast carcinoma with prognosis may in part be attributable to methodological variability, the location of infiltrating cell subsets within the tumor, the relative numbers of inflammatory cell subsets and their ensuing cytokine production, likely determine patient outcome. Indeed, the presence of intratumoral CD3+ TIL in ovarian carcinoma independently predicts progression-free and overall survival [193]. Likewise, the presence of CD8+ TIL at the invasive border of endometrial carcinoma independently predicts improved overall patient survival [274].

The effectiveness of the anti-tumor immune response is also dependent on the balance of effector and regulatory T cell (T_{reg}) subsets as a high CD8+/CD25+FOX-P3+ T_{reg} TIL ratio associates with improved survival in ovarian carcinoma patients [195].

CD4+CD25+FOX-P3+ T_{reg}, capable of *in vitro* and *in vivo* suppression of tumor antigen specific T-cell immunity are specifically recruited to ovarian tumors via CCL22 and high T_{reg} numbers associate with decreased survival [275]. Recently, Wolf et al. (2005) demonstrated high bulk tumor FOX-P3 mRNA levels independently predicted poor progression-free and overall survival in ovarian cancer patients [276]. In breast carcinomas, a substantial proportion of CD4+ TIL exhibit a T_{reg} phenotype (CD4+CD25+CTLA-4+CD45RO+) [184]. These T cells are elevated intratumorally and in the draining lymph nodes and peripheral blood and secrete interleukin (IL)-10 and transforming growth factor (TGF)-β but not interferon (IFN)-γ in response to non-specific stimulation, and suppress the proliferation and IFN-γ secretion of non-specifically activated $CD4^+CD25^-$ and $CD8^+$ *in vitro* [184].

Associations of TIL with prognosis in breast carcinoma have generally been investigated in the absence of information on intratumoral cytokine levels. The cytokine milieu of the tumor microenvironment is comprised of cytokines produced by tumor cells, stromal cells and infiltrating inflammatory cells. Tumor associated T cells are known to produce immune-modulating cytokines, in response to *ex vivo* stimulation and *in situ* in breast tumors [170, 188, 191, 196, 197], yet the clinical significance of cytokine production in breast carcinoma lesions and its relationship to immune cell infiltration have not been clearly elucidated [183, 199]. In this study we have characterized the pattern of immune cell infiltration in breast carcinomas and obtained immune profiles by assessing relative bulk tumor mRNA levels of the T_H1-type cytokines (IL-2, IL-12 and IFN-γ), T_H2-type cytokine (IL-4), immunoregulatory cytokines (IL-10 and TGF-β1) and pro-inflammatory cytokines (IL-1β, IL-6, and tumor necrosis factor (TNF)-α). The

presence of immunoregulatory T cells was indirectly determined by assessing FOX-P3 mRNA levels. The interrelationships and clinical implications of these variables were determined to obtain a comprehensive evaluation of the *in situ* immune response in invasive breast carcinoma.

5.3 MATERIAL AND METHODS

5.3.1 Tumor samples and Study subjects

One-hundred and twelve primary and 16 recurrent breast tumor lesions were randomly selected, based on tissue availability, from a larger sample with prognostic and outcome information, obtained from the Manitoba Breast Tumor Bank (MBTB) (Winnipeg, Manitoba, Canada), with approval of the local Human Investigation Committees. Tumor samples were assessed by immunohistochemistry (IHC) for immune cell infiltration and reverse-transcriptase polymerase chain reaction (RT-PCR) for cytokine and FOX-P3 mRNA levels. In total, 77 primary and 8 recurrent tumors were examined by immunohistochemistry and RT-PCR, 27 primary and 7 recurrent tumors were assessed by IHC only and 8 primary and one recurrent tumor had only RT-PCR information. A section of each tissue sample was stained with hematoxylin and eosin and following exclusion of samples where tumor tissue comprised <10% of the section area, immunohistochemical information was available for 99 primary and 15 recurrent lesions.

As several biological changes mark progression to metastatic disease, associations of immune cell infiltrate and relative mRNA levels with prognosis and survival were assessed for primary lesions only. Within the primary tumors, information on histological type was available for 108 tumors, of which most were infiltrating ductal (IDC, n=85),

with 20 infiltrating lobular (ILC) and 3 mixed IDC+ILC. Tumors spanned a wide range of grade (grades 5–9), determined for 103 tumors, using the Nottingham grading system [6] and were classified as I (score 5, n=15), II (scores 6-7, n=51) and III (scores 8-9, n=37). Tumor size was available for 109 tumors, categorized as small (≤2 cm, n=28) or large (>2 cm, n=81). Clinical lymph node (LN) status was available for all but one patient and 56 were LN+, with an average of 15 LN assessed per case (Mean ± SEM = 14.9 ± 1.5). One-hundred and eight tumors were staged according to AJCC guidelines [8], of which 16 were Stage I, 71 were Stage II, 18 were Stage III and 3 were Stage IV. The age at diagnosis was available for all patients and ranged from 32 to 86 years (median = 60 years; mean±sd = 59.3±14.5 years). Estrogen receptor (ER) and progesterone receptor (PR) levels were determined by ligand binding assay for all primary tumors and values ranged from 0 to 331 fmol/mg (median = 13.3 fmol/mg; mean±sd = 35.4±57.7 fmol/mg) and 0 to 1088 fmol/mg (median = 14.7 fmol/mg; mean±sd = 59.6±138.0 fmol/mg), respectively. Using a cutoff for negativity of less than 10fmol/mg, 48 were ER- and 73 were PR-. Her-2/neu expression was assessed in 89 tumors, as described below, and 19 (21.3%) tumors over-expressed Her-2/neu. The median follow-up time was 59 months (mean±sd = 57±30 months; range 2-127 months), during which 34 patients died of breast cancer. The median time to recurrence was 51 months (mean±sd = 49.2±32.1 months; range 0-127 months), during which 45 patients experienced recurrences (32 distant, 8 regional and 5 distant+regional).

5.3.2 Monoclonal Antibodies

Commercially available mAb (BD Biosciences Pharmingen, Mississauga, ON, Canada) were used to assess tumor cell expression of Her-2/neu (clone CB11, 1/100;

clone N12, 2μg/ml, Neomarkers, Quebec, Canada) and infiltrating $CD3^+$ TIL (clone UCHT1, 2.5μg/ml, Pharmingen), $CD4^+$ TIL (clone RPA-T4, 0.625μg/ml, Pharmingen), $CD8^+$ TIL (clone HIT8a, 0.625μg/ml, Pharmingen), $CD20^+$ B lymphocytes (clone HI(FB1), 2.5μg/ml, Pharmingen), $CD56^+$ NK cells (clone B159, 2.5μg/ml, Pharmingen) and $CD68^+$ TAM (clone EBM11, 2.15μg/ml, DakoCytomation, Mississauga, ON, Canada) cells. Tumor cells were identified using anti-cytokeratin mAb (clone AE1/AE3, 5μg/ml, DakoCytomation). Negative controls consisted of isotype matched non-specific mouse immunoglobulins (Southern Biotechnology Associates, Inc., Birmingham, AL, USA).

5.3.3 Immunohistochemistry

Serial frozen sections (8 μm) were fixed in acetone for 10 minutes at –20°C, shipped from the MBTB and stored at –70°C until immunohistochemical staining. After thawing, drying and rehydrating in phosphate buffered saline (PBS) (pH 7.4), sections were treated with 1.5% hydrogen peroxide in PBS for 30 minutes to remove endogenous peroxidases, and nonspecific binding was blocked with 15% goat serum in PBS for 1 hour. Sections were incubated for 1 hour with primary antibody followed by incubation for 30 minutes with goat anti-mouse DAKO EnVision horseradish peroxidase labeled polymer (DAKO Diagnostics Canada Inc., Mississauga, ON, Canada). Antibody binding was visualized by incubating with diaminobenzidine and hydrogen peroxide (Sigma, Oakville, ON, Canada) for 5 minutes. The reaction was stopped with water and sections were counterstained in Mayer's hematoxylin. Hematoxylin and eosin staining was

performed on one section for each breast carcinoma and samples where tumor cells comprised <10% of the section area were excluded from analysis.

5.3.4 Immunohistochemistry Interpretation

All slides were coded and independently examined by two readers, in the absence of information on prognostic parameters. Infiltrating CD3+, CD4+, CD8+, CD20+, CD56+ and CD68+ cells were coded based on estimated numbers: - (no or a few scattered cells); -/+ (small numbers of scattered cells or occasional small aggregates); + (moderate numbers of scattered cells, numerous small aggregates or occasional large aggregates); ++ (large numbers of scattered cells or several large aggregates). As the CD4 antigen is also expressed at lower levels on macrophages, to ensure CD4+ TIL specific coding, sections stained with anti-CD4 were compared to anti-CD3 and anti-CD68 stained sections. The patterns of immune cell infiltrate were recorded as focal (F) and/or diffuse (D) and the relative proportion of infiltrating CD3+ TIL and CD68+ TAM located intratumorally within tumor nests or within tumor stroma was also assessed.

Tumor cell expression of Her-2/neu was determined in 89 primary and 13 recurrent tumors and coded as 0 (<10% with membrane staining), 1+ (≥ 10% with weak, incomplete membrane staining), 2+ (≥ 10% with weak to moderate complete membrane staining) or 3+ (≥ 10% with strong complete membrane staining). As some tumors coded 2+ by IHC will not show gene amplification by fluorescent in situ hybridization [249], only those tumors coded 3+ were considered clinically positive for analysis.

5.3.5 RNA extraction, and semi quantitative RT-PCR

Total RNA was isolated from breast tumor tissues by homogenization using Trizol reagent (Gibco BRL, Rockville, MD), followed by treatment with DNA-free reagent (Ambion, Austin, TX) to remove any contaminating DNA. Reverse transcription was performed on 1 μg RNA using the First Strand cDNA Synthesis Kit (Pharmacia Biotech, Quebec, Canada). PCR was performed using a Biometra T Gradient thermocycler (Montreal Biotech Inc., Quebec, Canada) to amplify cDNA using primers described in Supplementary Table 5S1. All PCR reactions were performed in a volume of 50 μL with 200μM dNTPs (Gibco BRL) and 1 μL cDNA. PCR buffer contained 20mM Tris-HCl (pH 8.4) and 50 mM KCl. Samples containing water instead of test cDNA were included as contamination controls and cDNA from the cell lines Jurkat E6-1 (CD3, IL-2, IL-4, IL-10, TGF-β), C10/MJ (IFN-γ, IL-6, FOX-P3), U937 (TNF-α, IL-1β) and YAR (IL-12) were used as positive controls. Primers were synthesized by Gibco BRL and included β-actin [251], CD3, IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-12, TNF-α [252], IL-1β [253], TGF-β1 [254] and FOX-P3 [255]. Primers were used at concentrations of 20 pM for β-actin, IL-1β, IL-6, TNF-α and FOX-P3 and 10 pM for all others. $MgCl_2$ (Gibco BRL) concentration was 1.5 mM for β-actin, IL-4 and FOX-P3 and 2 mM for all other reactions. 0.2 μL of Taq DNA polymerase (Gibco BRL) was used for β-actin, IL-1β, IL-6 and FOX-P3 reactions and all other reactions used 0.25 μL. Reaction mixtures were amplified for 35 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 55°C (IFN-γ and IL-2), 72°C (TNF-α) or 65°C (all others), and extension at 72°C for 1 min, followed by a separate 5 min extension step at 72°C for all but IL-1β, IL-6 and FOX-P3.

The intensity of amplified products was semi-quantified and normalized as a percent of β-actin using the ChemiImager 4000 with Alphaease 4.0 Software.

5.3.6 Statistical Analysis

Contingency tables were analyzed using Pearson's chi-square analysis or Fisher's exact two-sided t-test for 2x2 tables with expected counts ≤ 5. Non-parametric Mann-Whitney (2 category variables) and Kruskal-Wallis (≥2 category variables) were used when assessing statistical significance of continuous variables. Infiltrating inflammatory cell categories and relative amounts of CD3, cytokine and FOX-P3 mRNA were compared using Spearman's rank order correlation.

Survival estimates were calculated using Kaplan-Meier method with log-rank statistic. Ninety-five percent confidence intervals (95% CI) around 5-year percent survival estimates were calculated using the standard error (SE) of the cumulative survival probability (95%CI = cumulative survival probability ±1.96 x SE). Estimates were calculated as time to regional or distant metastasis for recurrence-free survival (RFS), time to distant metastasis for distant recurrence-free survival (dRFS) and time to death from breast cancer for disease-specific survival (DSS). For disease-free survival, patients who died of other causes or who were alive with other malignancy were censored from analysis.

Backward stepwise Cox proportional hazards models were constructed for multivariate survival analyses, using a probability of $P<0.05$ for stepwise entry and $P>0.06$ for removal. No patients were lost to follow-up and patients not experiencing the event were censored at the time of last follow-up or at 5 years for 5-year survival. For survival analysis, normalized cytokine and FOX-P3 units were stratified into high and

low categories by comparing the 4th quartile to the 1st-3rd quartiles. Relative mRNA levels were also entered into univariate Cox regression analysis as continuous covariates to ensure no loss of data attributable to the categorization. To determine if the balance of relative mRNA levels influence patient survival, mRNA levels were ranked and ratios of ranked mRNA were computed. Again, tumors were stratified into high and low ratio categories using the 75th percentile of each rank ratio. For multivariate analysis, correlation matrices were constructed to ensure lack of collinearity of covariates. For all statistical tests, differences between groups were considered significant if $P<0.05$. All analysis was performed using SPSS Version 11.5 statistical software.

5.4 RESULTS:

5.4.1 Characterization of the immune cell infiltrate and cytokine milieu in invasive breast carcinoma lesions

Infiltrating inflammatory cell subsets were assessed in 99 primary and 15 recurrent breast carcinoma lesions, via indirect immunohistochemistry. Information on infiltrating CD3+ TIL and CD68+ TAM were available for all tumors. Both subsets were detected intratumorally and in surrounding stroma and representative staining patterns are depicted in Figure 5.1. Due to lack of tissue availability or tissue loss during staining procedure, infiltrating CD20+ cells were assessed in 98 primary tumors and all recurrent lesions and CD4+ and CD8+ TIL were evaluated in 97 primary and 14 recurrent lesions. All tumors had CD68+ infiltrating cells and the majority contained infiltrating CD3+, CD4+ and CD8+ cells. In contrast, CD20+ cells occurred in smaller numbers. Infiltrating CD56+ cells were evaluated in a subset of tumors (43 primary and 8 recurrent) and were

infrequent. An increased proportion of recurrent lesions lacked CD8+ TIL as compared to primary breast tumors (P=0.040) (Supplementary Table 5S2). Within primary tumors, positive correlations were observed between infiltrating CD3+, CD4+, CD8+, CD20+ and CD68+ cell populations (Table 5.1).

Tumors with large numbers of TIL and TAM had elevated proportions of CD3+ TIL and CD68+ TAM located intratumorally (Supplementary Figure 5S1). The distribution of TIL and TAM was also dependent on relative numbers of infiltrating cells. Infiltrating immune cells were distributed focally and/or diffuse throughout the tumor mass (Supplementary Figure 5S2). Small numbers of CD3+, CD4+ and CD8+ TIL were primarily focally aggregated in the tumor mass, while large numbers were distributed throughout the tumor. CD20+ B cells were relatively infrequent and were predominantly focally distributed. In contrast, CD68+ TAM were diffusely distributed throughout the tumor mass (Supplementary Figure 5S3). CD56+ NK cells were detected in only 2/43 tumors examined and in both cases were focally distributed (data not shown).

Relative cytokine, CD3 and FOX-P3 mRNA levels were assessed in primary and recurrent lesions using RT-PCR (Supplementary Table 5S3). All tumors had detectable TGF-β1 and the majority contained CD3, IFN-γ, IL-2, IL-12, IL-10, FOX-P3, IL-1β and IL-6 transcripts. Relative levels of IL-4 and TNF-α mRNA were decreased in comparison. IL-12 was decreased in recurrent tumors as compared to primary tumors (P=0.030). Other cytokines and FOX-P3 did not significantly differ between primary and recurrent lesions. In total, 60 primary tumors had information on CD3, all cytokines and

Figure 5.1: Representative immunohistochemistry examples of infiltrating CD3+ and CD68+ cells in primary breast tumors. Indirect immunohistochemistry was used to assess infiltrating CD3+ TIL (mAb UCHT1) and CD68+ TAM (mAb EBM11) in invasive breast carcinoma lesions. Hematoxylin and eosin staining illustrates invasive ductal morphology **(A1-D1)**. Isotype control antibodies were included as a negative control **(A2-D2)** and tumor cells were identified with anti-cytokeratin (mAb AE1/AE3) **(A3-D3)**. These representative examples depict tumors that were coded for infiltrating CD3+ cells as none **(A4)**, small numbers **(B4)**, moderate numbers **(C4)**, and large numbers **(D4)**. These tumor samples were infiltrated by small **(A5)**, moderate **(C5)** and large **(B5, D5)** numbers of CD68+ cells, respectively.

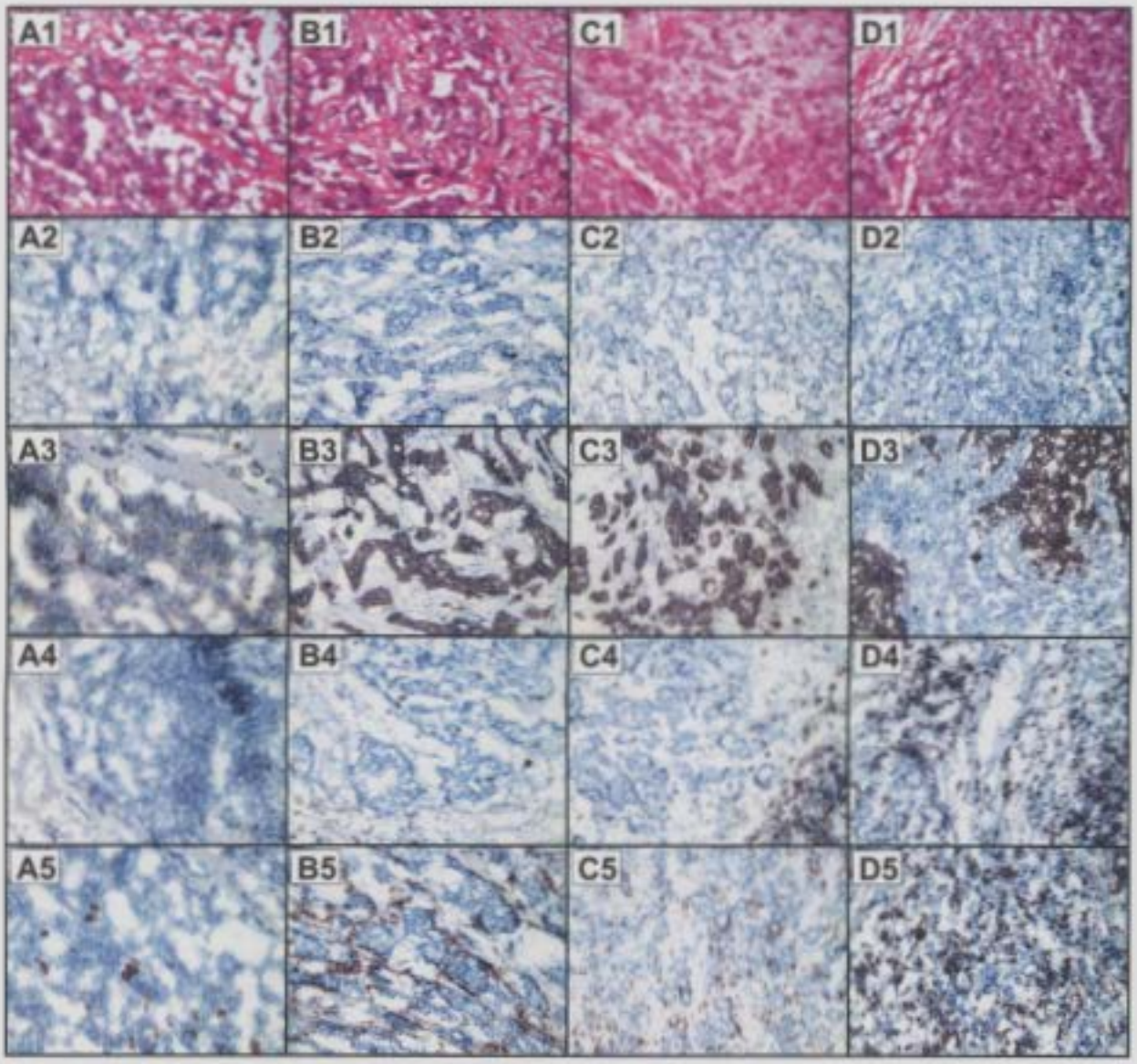

A1
B1
C1
D1
A2
B2
C2
D2
A3
B3
C3
D3
A4
B4
C4
D4
A5
B5
C5
D5

Table 5.1: Spearman's correlation coefficients[†] for infiltrating inflammatory cell subsets in primary invasive breast carcinoma.

Infiltrate	Infiltrate			
	CD3+	CD4+	CD8+	CD20+
CD3+	1	-	-	-
CD4+	**0.88****	1	-	-
CD8+	**0.87****	**0.76****	1	-
CD20+	**0.48****	**0.50****	**0.46****	1
CD68+	**0.53****	**0.57****	**0.46****	**0.37****

[†]Listwise comparison of primary breast tumors (N=96).
** Correlation is significant at the 0.01 level (2-tailed).

FOX-P3 (Table 5.2). Levels of CD3 positively correlated with the T_H1 cytokines, FOX-P3 and IL-6. Positive correlations were observed between the T_H1 cytokines, IFN-γ, IL-2 and IL-12; the T_H2/T_H3 cytokines, IL-4 and IL-10 and the pro-inflammatory cytokines IL-1β and IL-6. Positive correlations were also observed between IL-10 and IL-12, IL-1β and TGF-β, while IL-6 positively correlated with IL-2 and IL-12. FOX-P3 mRNA also positively correlated with levels of T_H1 cytokines and with the pro-inflammatory cytokines, IL-1β and IL-6.

5.4.2 Infiltrating inflammatory cells associate with the in situ cytokine milieu and CD3 and FOX-P3 mRNA in primary breast tumors

As differences were observed between primary and recurrent lesions, to discount any immunological changes that coincide with metastasis development, associations of infiltrating cell subsets and relative cytokine mRNA levels were compared within primary tumors. Since TIL were sometimes focally distributed throughout the tumor, relative levels of CD3 mRNA were also assessed to ensure the bulk tumor RNA was derived from an area representative of the region of tumor assessed for TIL by IHC. By Spearman's correlation, relative levels of CD3 mRNA correlated with increasing numbers of CD3+ TIL (ρ=0.37, P=0.008), CD4+ TIL (ρ=0.41, P=0.003), and CD8+ TIL (ρ=0.37, P=0.020), but not CD20+ (ρ=0.17, P=0.242) or CD68+ (ρ=-0.09, P=0.529) infiltrating cell populations.

Relative levels of cytokine and FOX-P3 mRNA were compared in tumors stratified by numbers of infiltrating inflammatory cells. To obtain a comprehensive view of the immune profile in these breast carcinoma lesions, analysis was performed on only

Table 5.2: Spearman's correlation coefficients for relative *in situ* CD3, cytokine and FOX-P3 mRNA amounts[†] in breast carcinoma.

		T_H1/cellular immunity			$T_H2/T_H3/T_{reg}$				Pro-inflammatory		
	CD3	IFN-γ	IL-2	IL-12	IL-4	IL-10	TGF-β	FOX-P3	IL-1β	IL-6	TNF-α
CD3	1	-	-	-	-	-	-	-	-	-	-
IFN-γ	**0.74****	1	-	-	-	-	-	-	-	-	-
IL-2	**0.73****	**0.75****	1	-	-	-	-	-	-	-	-
IL-12	**0.55****	**0.56****	**0.65****	1	-	-	-	-	-	-	-
IL-4	-0.04	0.02	0.06	0.15	1	-	-	-	-	-	-
IL-10	0.21	0.20	0.22	**0.55****	**0.27***	1	-	-	-	-	-
TGF-β	0.06	-0.20	-0.06	0.01	-0.01	0.15	1	-	-	-	-
FOX-P3	**0.38****	**0.40****	**0.35****	**0.40****	0.16	0.11	-0.04	1	-	-	-
IL-1β	0.23	0.001	0.16	0.22	0.13	0.07	**0.33****	**0.39****	1	-	-
IL-6	**0.39****	0.24	**0.30***	**0.30***	0.01	0.15	0.18	**0.43****	**0.59****	1	-
TNF-α	0.10	0.19	0.17	0.24	0.02	0.06	-0.01	0.20	0.02	0.05	1

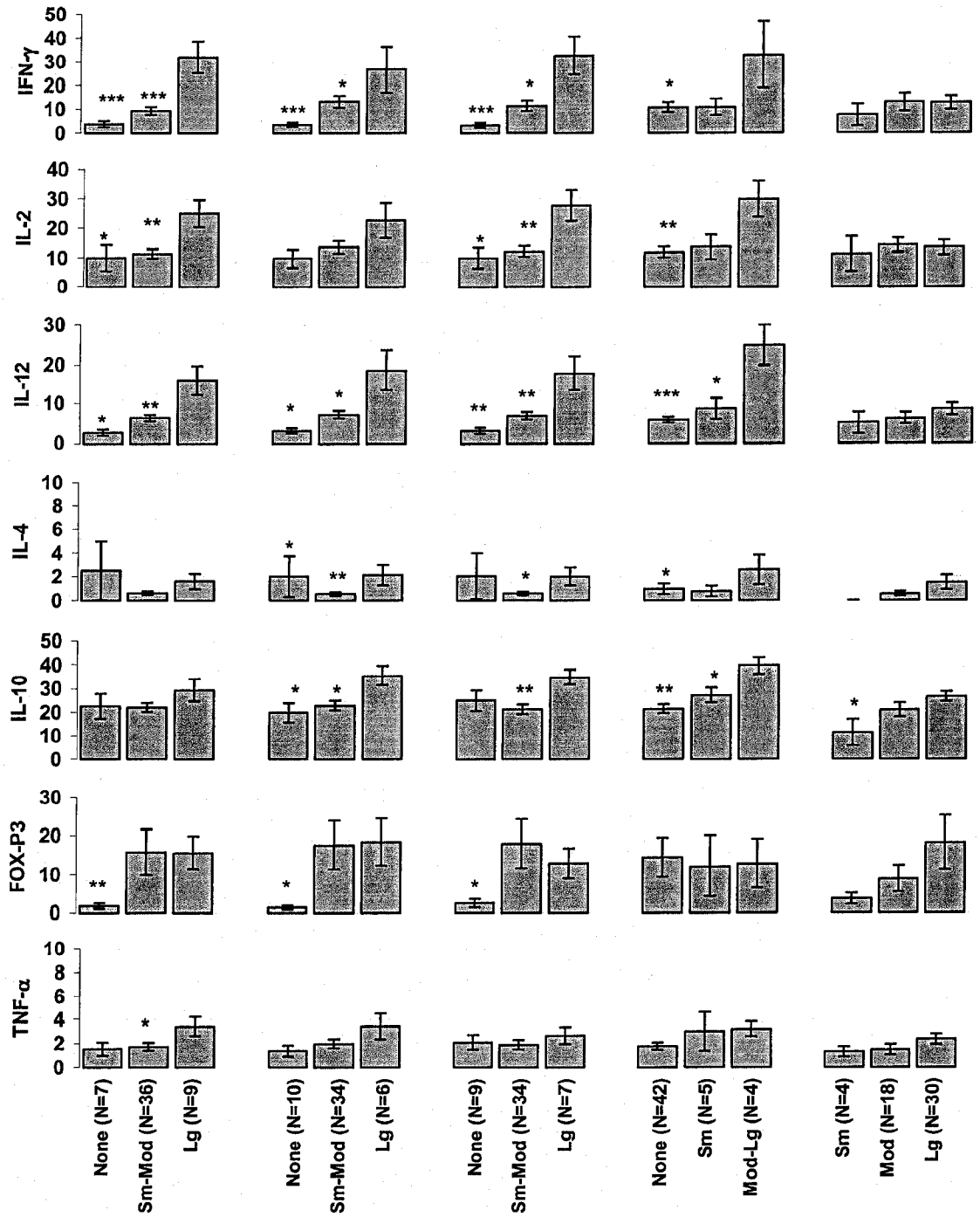
[†]Listwise comparison of primary breast tumors (N=60).

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

those tumors with complete mRNA profiles. For CD3+, CD4+ and CD8+ TIL, relative mRNA levels did not differ between those with small and moderate numbers (data not shown), thus both groups were grouped for comparison. For CD20+ B cells, only one tumor contained large numbers, thus moderate and large numbers were grouped for comparison. As shown in Figure 5.2 tumors with large numbers of CD3+ TIL had significantly increased levels of the T_H1-type cytokines IFN-γ, IL-2 and IL-12 mRNA, reflected by both $CD4^+$ and $CD8^+$ cell populations. IL-4 was also increased in tumors with large numbers of $CD4^+$ cells or $CD8^+$ cells as compared to those with small to moderate numbers and tumors with CD3+, CD4+ and CD8+ TIL had significantly higher FOX-P3 mRNA levels. Relative levels of TNF-α mRNA were increased in tumors with large numbers of CD3+ TIL, reflected mainly by the CD4+ subset. Tumors with moderate to large numbers of CD20+ cells had increased levels of both T_H1-type cytokines, IFN-γ, IL-2 and IL-12 and the T_H2-type cytokines IL-4 and IL-10. Tumors with large numbers of CD68+ TAM had significantly higher IL-10 levels as compared to those with small numbers. Relative amounts of TGF-β, IL-1β, and IL-6 did not significantly associate with infiltrating inflammatory cell subsets (data not shown). Performing the above analysis on all informative samples yielded similar results (not depicted). Reflecting the associations with large numbers of infiltrating cells, those tumors in which the relative proportion of intratumoral TIL was equivalent to or exceeded stromal TIL had significantly higher levels of T_H1-type cytokines (IL-2, IL-12 and IFN-γ), IL-10, FOX-P3 and IL-6. Likewise, levels of IL-10 were significantly elevated in tumors where the proportion of intratumoral TAM was greater than or equivalent to that of stromal TAM (data not shown).

Figure 5.2: Associations of tumor infiltrating inflammatory cells with relative in situ cytokine and FOX-P3 mRNA levels. Levels of intratumoral mRNA were compared in breast tumors categorized by infiltrating cell populations. Relative numbers of infiltrating cell subsets were determined using indirect immunohistochemistry as defined in Methods. RT-PCR was carried out using total RNA (1 μg) prepared from fresh-frozen breast carcinoma tissue. Relative amounts of cytokine mRNA were normalized to β-actin and averaged (1-3 RT-PCR reactions) for each breast carcinoma sample. Bars represent average of all breast tumors in each infiltrate subgroup ± standard error of the mean. Associations of infiltrating cell subsets with relative mRNA levels were determined using Kruskal-Wallis H test and P-values for 3 category comparisons are provided in the table. Asterisks indicate statistically significant differences in comparison to large numbers of infiltrating cells assessed using Mann-Whitney U test (*P<0.05, **P<0.01, ***P<0.001).



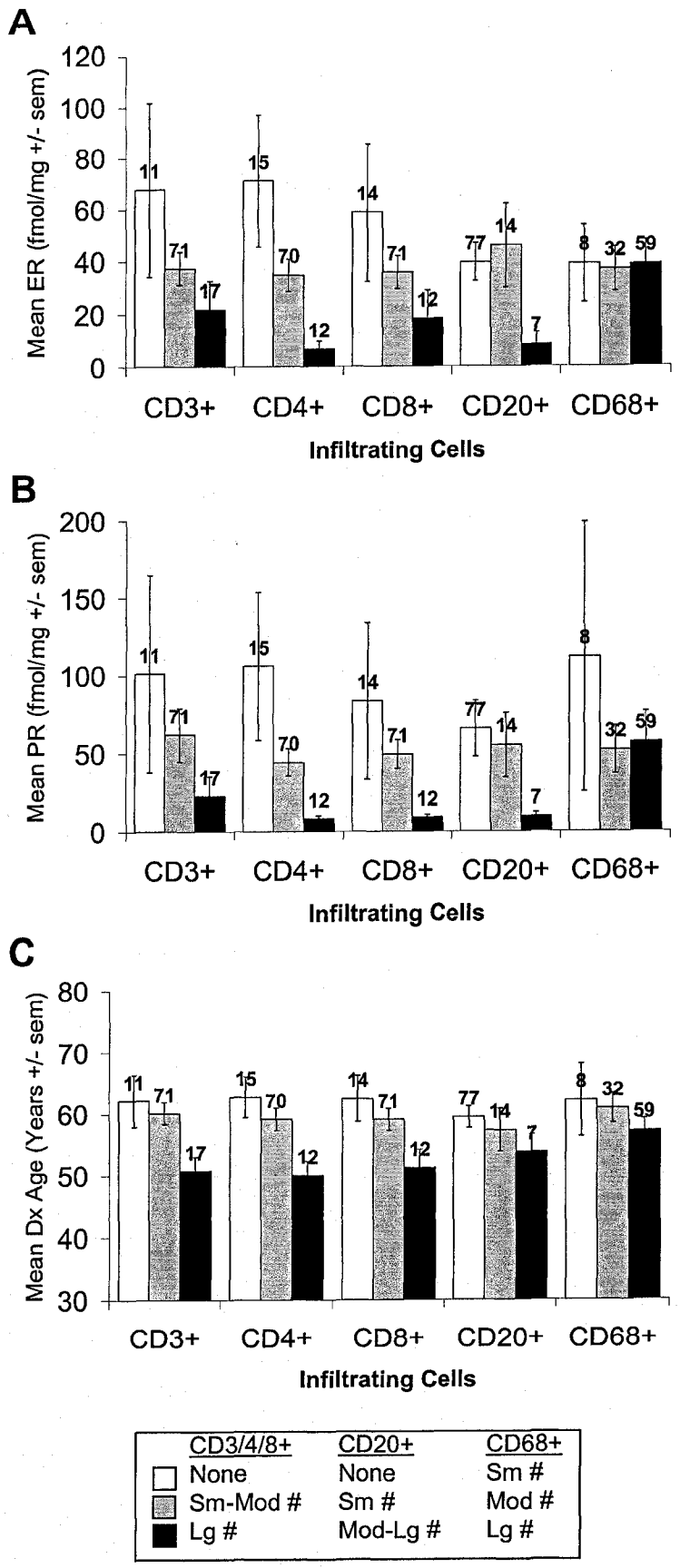
	CD3+	CD4+	CD8+	CD20+	CD68+
IFN-γ	**0.001**	**0.004**	**0.001**	0.067	0.775
IL-2	**0.010**	0.108	**0.011**	**0.030**	0.670
IL-12	**0.002**	**0.003**	**0.002**	**0.004**	0.502
IL-4	0.108	**0.018**	**0.032**	0.058	0.169
IL-10	0.301	**0.047**	**0.029**	**0.022**	0.051
FOX-P3	**0.030**	**0.005**	**0.042**	0.784	0.302
TNF-α	0.081	0.206	0.407	0.195	0.313

5.4.3 Associations of infiltrating cells, and relative cytokine and FOX-P3 mRNA levels with prognostic indicators in breast carcinoma

Associations of infiltrating cell subsets with clinicopathological parameters were assessed within primary tumors. Infiltrating CD3+ TIL associated with decreased ER, PR and diagnosis age and increased tumor grade, reflected by both CD4+ and CD8+ populations (Figure 5.3A-C). CD20+ infiltrating cells did not significantly associate with ER, PR or age at diagnosis, but positively associated with tumor grade (Figure 5.3D). Infiltrating CD68+ cells did not associate with ER, PR or diagnosis age but CD68+ infiltrate associated with increased histological grade (Figure 5.3D) and lymph node metastasis (Figure 5.3E). Infiltrating cell subsets did not significantly associate with tumor diameter (Figure 5.3F), TNM stage, tumor type or Her-2/neu over-expression (data not shown).

Relative cytokine and FOX-P3 mRNA levels were compared in tumors stratified by categorical prognostic parameters (Table 5.3). IDC tumors had elevated levels of IFN-γ and decreased IL-1β and TGF-β1. Large numbers of TIL display only a trend for decreased tumor size, however, tumors with a small diameter (≤2 cm) had elevated levels of the elevated levels of CD3 mRNA and the T_H1 cytokines, IFN-γ and IL-2 and display a trend for elevated IL-12. Paralleling the association of inflammatory infiltrate with tumor grade, levels of the pro-inflammatory cytokines IFN-γ and TNF-α were increased in poorly differentiated Grade III tumors. Over-expression of Her-2/neu did not associate with immune cell infiltration or T cell specific cytokine production but negatively associated with TGF-β1 (P=0.018) and IL-1β (P=0.029). Relative mRNA levels did not

Figure 5.3: Association of infiltrating T cell subsets with prognostic parameters in breast carcinoma patients. **A)** ER negatively associates with infiltrating CD3+ (P=0.054), CD4+ (P=0.003), and CD8+ (P=0.056) cells, but not CD20+ (P=0.267) or CD68+ (P=0.089) cells. **B)** PR negatively associates with infiltrating CD3+ (P=0.015), CD4+ (P=0.003), and CD8+ (P=0.024) cells, but not CD20+ (P=0.153) or CD68+ (P=0.474) cells. **C)** Diagnosis age is lower in patients with tumor infiltrating CD3+ (P=0.036), CD4+ (P=0.062), and CD8+ (P=0.122) cells, but not CD20+ (P=0.564) or CD68+ (P=0.414) cells. **D)** All tumor infiltrating cell populations associate with histological grade III (CD3+ (P=0.001), CD4+ (P=0.003), CD8+ (P=0.004), CD20+ (P=0.009), CD68+ (P=0.017). **E)** Infiltrating CD68+ cells associate with LN metastasis (P=0.024), but other infiltrating cell populations do not associate with LN metastasis (CD3+ (P=0.906), CD4+ (P=0.299), CD8+ (P=0.470), CD20+ (P=0.185)). **F)** Tumor diameter did not associate with infiltrating cell populations (CD3+ (P=0.339), CD4+ (P=0.142), CD8+ (P=0.142), CD20+ (P=0.537), CD68+ (P=0.224)). Numbers at end of bars represent N values for each category. Variables were analyzed using one-way ANOVA for diagnosis age, Kruskal-Wallis H test for ER and PR and Pearson's Chi-square test for categorical variables.


A
Mean ER (fmol/mg +/- sem)
120
100
80
60
40
20
0
11
71
17
15
70
12
14
71
12
77
14
7
8
32
59
CD3+
CD4+
CD8+
CD20+
CD68+
Infiltrating Cells
B
Mean PR (fmol/mg +/- sem)
200
150
100
50
0
11
71
17
15
70
12
14
71
12
77
14
7
8
32
59
CD3+
CD4+
CD8+
CD20+
CD68+
Infiltrating Cells
C
Mean Dx Age (Years +/- sem)
80
70
60
50
40
30
11
71
17
15
70
12
14
71
12
77
14
7
8
32
59
CD3+
CD4+
CD8+
CD20+
CD68+
Infiltrating Cells
CD3/4/8+
None
Sm-Mod #
Lg #
CD20+
None
Sm #
Mod-Lg #
CD68+
Sm #
Mod #
Lg #

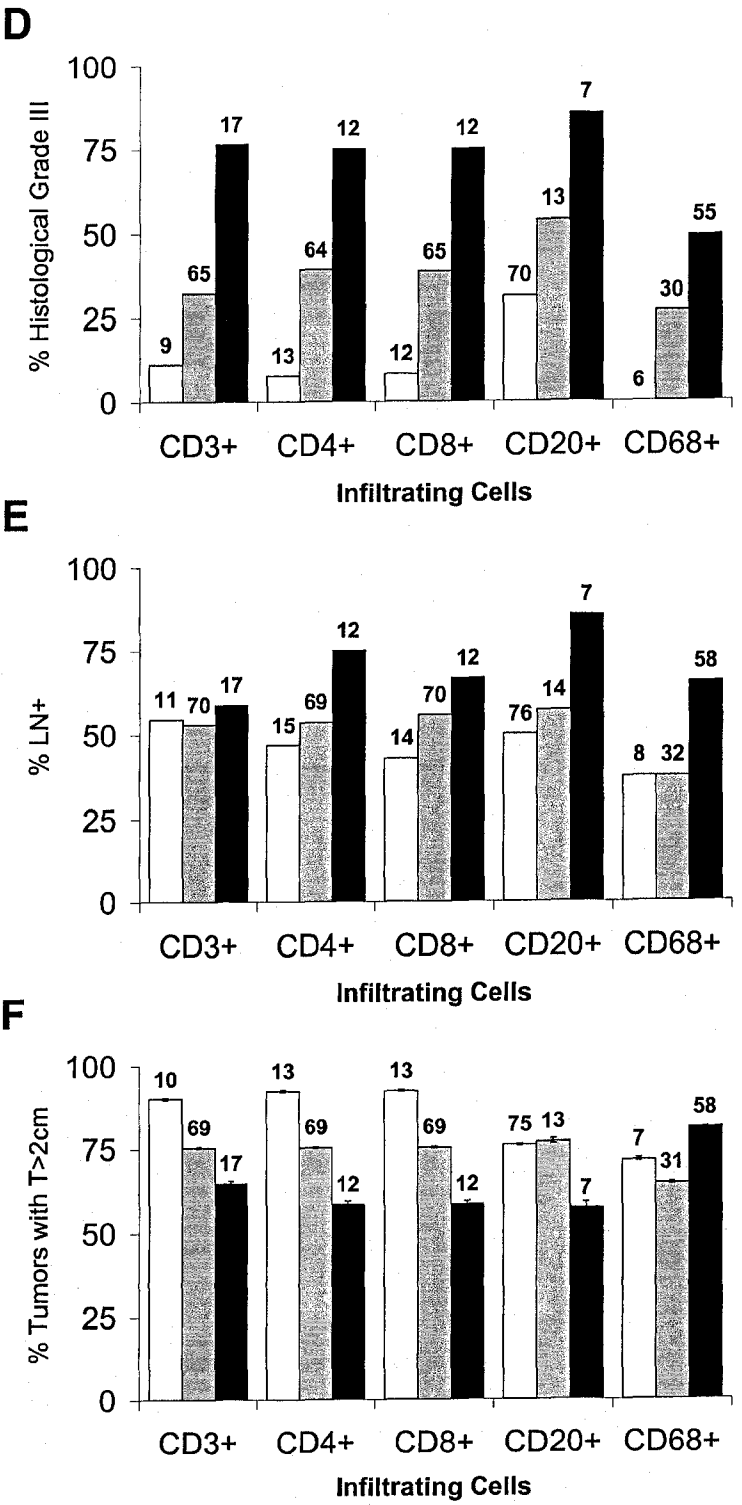


Figure 5.3 continued.

associate with categorical LN status, TNM stage, and ER, PR or diagnosis age. Spearman's correlation analysis of continuous prognostic variables with relative mRNA levels identified negative associations between diagnosis age and CD3 mRNA (ρ=-0.241, P=0.030; N=81), ER and FOX-P3 (ρ=-0.248, P=0.029; N=77) and tumor diameter negatively correlated with IFN-γ (ρ=-0.229, P=0.041; N=80) and IL-2 (ρ=-0.232, P=0.040; N=79).

5.4.4 Relative numbers of CD3+ and CD8+ TIL predict patient survival

Despite the association of infiltrating cell subsets with poor prognostic indicators (Figure 5.3), univariate testing did not demonstrate significant associations of infiltrating cell subsets, or their distribution and location within the tumor, with recurrence-free survival (RFS), distant RFS (dRFS), or disease-specific survival (DSS) (data not shown). However, a trend was observed for decreased dRFS of patients with tumors containing small to moderate numbers of CD3+ TIL. This was reflected by the CD8+ TIL subset but not the CD4+ TIL subset (Figure 5.4). Cox proportional hazards models were constructed and analyzed using the backward stepwise method, to test the ability of inflammatory cell subsets to independently predict patient survival, when controlling for standard prognostic indicators. In addition to advanced TNM stage and PR-negative tumors, the presence of small to moderate numbers of both CD3+ TIL and CD8+ TIL but not CD4+ TIL independently predicted earlier time to distant recurrence (Table 5.4). Infiltrating TIL subsets did not independently predict RFS or DSS and CD20+ B cell and CD68+ TAM categories did not associate with RFS, dRFS or DSS (data not shown). Neither Her-2/neu expression status nor tumor grade were included in the above models as inclusion

Table 5.3: Associations of relative intratumoral cytokine and FOX-P3 mRNA levels with prognostic parameters in breast carcinoma

	Mean Relative mRNA ± SEM (N)										
		T_H1			$T_H2/T_H3/T_{reg}$				Pro-inflammatory		
	CD3	**IL-2**	**IL-12**	**IFN-γ**	**IL-4**	**IL-10**	**TGF-β**	**FOX-P3**	**IL-1β**	**IL-6**	**TNF-α**
IDC	34.7±2.6 (63)	12.2±1.5 (62)	7.4±1.0 (58)	11.2±1.8 (64)	1.3±0.4 (59)	22.9±1.6 (58)	53.0±3.2 (54)	14.3±4.0 (59)	30.0±4.9 (59)	30.2±3.0 (59)	1.9±0.3 (53)
ILC or mixed	30.1±5.0 (16)	10.7±2.1 (17)	6.8±1.5 (15)	4.9±1.9 (16)	1.1±0.4 (15)	17.3±4.0 (15)	60.1±4.6 (13)	28.3±12.3 (16)	42.6±7.8 (16)	48.4±9.5 (16)	1.8±0.8 (16)
P-value	0.495	0.981	0.946	**0.045**	0.649	0.112	0.067	0.476	**0.047**	0.095	0.209
Grade I + II	31.1±2.9 (51)	10.4±1.2 (52)	6.2±0.9 (48)	7.5±1.4 (52)	1.2±0.4 (49)	20.6±1.9 (48)	53.6±3.6 (43)	16.5±4.8 (49)	31.2±3.5 (49)	36.6±4.4 (49)	1.4±0.3 (49)
Grade III	38.3±4.3 (25)	13.7±2.7 (24)	9.5±1.9 (22)	15.2±3.6 (25)	1.5±0.6 (22)	23.2±2.5 (22)	55.7±5.0 (21)	14.4±6.8 (23)	35.8±11.6 (23)	28.7±3.6 (23)	3.3±0.6 (17)
P-value	0.143	0.554	0.137	0.081	0.744	0.379	0.864	0.235	0.695	0.651	**0.001**
LN-	33.7±3.7 (40)	10.6±1.5 (41)	6.0±0.8 (38)	9.3±1.9 (41)	1.0±0.3 (38)	20.9±2.3 (38)	52.6±4.0 (34)	15.6±5.4 (39)	37.4±7.6 (39)	30.0±4.4 (39)	2.0±0.4 (36)
LN+	33.8±2.8 (40)	12.9±2.0 (39)	8.2±1.4 (36)	10.6±2.3 (40)	1.4±0.5 (37)	23.1±2.0 (36)	56.4±3.8 (34)	19.4±6.2 (37)	28.0±3.0 (37)	38.7±4.4 (37)	1.7±0.4 (34)
P-value	0.704	0.791	0.570	0.642	0.873	0.424	0.477	0.368	0.799	0.105	0.636
T ≤2cm	42.9±4.6 (20)	16.6±2.4 (19)	9.8±2.0 (20)	17.6±4.3 (20)	1.9±0.6 (20)	24.3±3.4 (20)	52.1±4.3 (18)	14.8±5.8 (19)	23.7±4.4 (19)	26.5±4.9 (19)	2.7±0.7 (18)
T>2cm	31.1±2.6 (59)	10.3±1.4 (60)	6.1±0.9 (53)	7.5±1.2 (60)	1.0±0.4 (54)	21.4±1.7 (53)	56.1±3.4 (49)	18.7±5.2 (56)	36.3±5.4 (56)	37.4±3.9 (56)	1.5±0.3 (51)
P-value	**0.026**	**0.008**	0.067	**0.028**	0.062	0.439	0.921	0.562	0.057	0.115	0.104
TNM Stage I-II	35.3±2.8 (63)	12.4±1.4 (64)	6.9±0.9 (58)	11.0±1.8 (64)	1.4±0.4 (58)	21.8±1.8 (58)	55.1±3.3 (53)	18.6±5.0 (58)	35.1±5.3 (58)	33.7±3.7 (58)	1.9±0.3 (56)

	Mean Relative mRNA ± SEM (N)										
		T_H1			$T_H2/T_H3/T_{reg}$				Pro-inflammatory		
	CD3	**IL-2**	**IL-12**	**IFN-γ**	**IL-4**	**IL-10**	**TGF-β**	**FOX-P3**	**IL-1β**	**IL-6**	**TNF-α**
TNM Stage III-IV	28.5±3.3 (15)	8.6±2.3 (14)	7.1±2.2 (14)	5.7±1.3 (15)	0.3±0.1 (15)	23.0±3.3 (14)	54.7±4.4 (13)	14.7±6.9 (16)	27.0±3.8 (16)	38.8±6.2 (16)	1.2±0.3 (12)
P-value	0.421	0.246	0.836	0.555	0.110	0.787	0.519	0.324	0.953	0.348	0.561
ER<10 fmol/mg	32.4±3.5 (32)	9.8±1.9 (31)	7.8±1.7 (27)	9.7±2.7 (32)	1.6±0.5 (27)	20.9±2.4 (27)	57.1±4.7 (23)	15.9±5.6 (28)	37.5±9.5 (28)	35.7±5.2 (28)	2.0±0.4 (27)
ER≥10 fmol/mg	34.7±3.0 (49)	13.1±1.6 (50)	6.8±0.9 (48)	10.2±1.7 (50)	1.0±0.4 (49)	22.7±1.9 (48)	53.2±3.3 (46)	18.2±5.5 (49)	29.9±3.5 (49)	33.2±3.9 (49)	1.8±0.4 (44)
P-value	0.757	0.099	0.947	0.404	0.700	0.581	0.441	0.165	0.427	0.649	0.462
PR<10 fmol/mg	38.0±4.0 (28)	13.8±2.4 (28)	8.0±1.7 (23)	11.6±3.0 (28)	1.1±0.5 (23)	20.1±2.6 (23)	51.7±4.2 (20)	18.8±7.5 (24)	38.8±11.1 (24)	39.5±6.3 (24)	1.8±0.5 (23)
PR≥10 fmol/mg	31.7±2.8 (53)	10.9±1.4 (53)	6.8±0.9 (52)	9.2±1.6 (54)	1.2±0.4 (53)	22.9±1.9 (52)	55.7±3.4 (49)	16.8±4.8 (53)	29.8±3.3 (53)	31.6±3.5 (53)	1.9±0.3 (48)
P-value	0.160	0.525	0.687	0.883	0.618	0.395	0.731	0.447	0.516	0.250	0.395
Dx Age<50 years	35.2±4.1 (24)	11.1±2.3 (24)	7.7±1.7 (23)	9.5±2.5 (24)	0.8±0.3 (23)	22.5±2.4 (23)	58.8±7.3 (20)	14.0±6.6 (23)	41.5±11.7 (23)	34.8±6.2 (23)	2.0±0.6 (19)
Dx Age≥50 years	33.3±2.8 (57)	12.2±1.4 (57)	6.9±0.9 (52)	10.2±1.8 (58)	1.4±0.4 (53)	21.9±1.9 (52)	52.8±2.4 (49)	18.8±5.0 (54)	28.8±3.1 (54)	33.8±3.6 (54)	1.8±0.3 (52)
P-value	0.552	0.518	0.931	0.967	0.846	0.756	0.926	0.482	0.322	0.929	0.833
Her-2/neu (Codes 0-2)	36.0±3.2 (48)	11.7±1.5 (49)	7.1±1.0 (43)	10.4±1.8 (49)	1.0±0.4 (44)	21.7±1.8 (43)	56.3±4.0 (41)	17.8±5.3 (45)	32.8±6.2 (45)	35.4±3.6 (45)	1.8±0.3 (39)
Her-2/neu (Code 3)	29.9±5.5 (15)	15.6±3.7 (15)	7.7±2.4 (15)	13.5±5.0 (15)	1.5±0.6 (15)	21.3±3.9 (15)	39.4±4.7 (13)	17.1±11.1 (13)	20.1±5.2 (13)	23.1±6.0 (13)	1.7±0.4 (15)
P-value	0.290	0.375	0.790	0.943	0.418	0.716	**0.018**	0.695	**0.029**	0.058	0.829

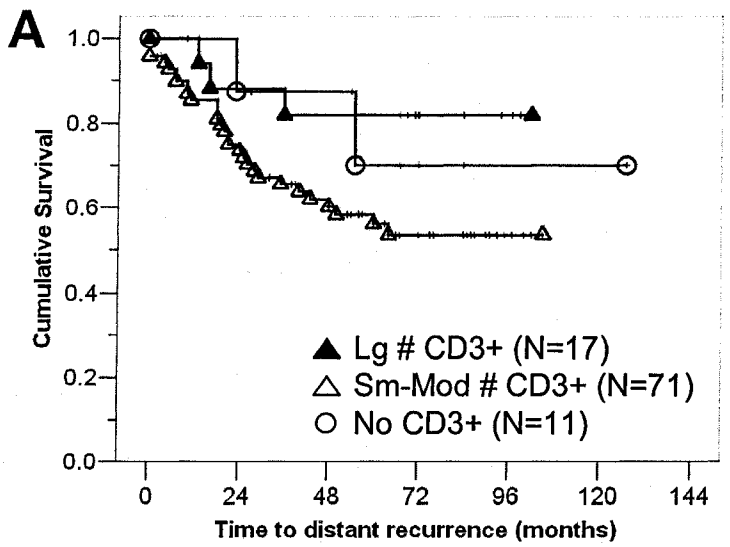
decreased sample size and did not alter the prognostic significance of the other covariates.

5.4.5 Elevated TNF-α within breast tumors predicts improved survival of breast carcinoma patients

We have recently reported that patients with tumors containing high relative levels of IFN-γ have improved RFS, and dRFS, by univariate survival analysis, while levels of IL-2, IL-12, IL-4, IL-10 and TGF-β did not associate with patient survival (Chapter 4) [256]. Here we have also analyzed relative mRNA levels of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and the T_{reg} marker FOX-P3. Tumors were stratified into high and low groups using the 75th percentile cut-point. In addition, as recommended by Altman et al. (1994) [277], to ensure there was no loss of information due to relative mRNA level categorization, all cytokine and FOX-P3 mRNA levels were entered separately as a continuous covariate into an univariate Cox regression analysis. Categories of IL-1β, IL-6 and FOX-P3 mRNA levels did not significantly associate with RFS, dRFS or DSS (data not shown). Furthermore, analysis of these and the previously studied cytokines (Chapter 4) as continuous covariates did not yield significant differences (data not shown).

With respect to TNF-α mRNA levels, although RFS did not differ in patients stratified by TNF-α levels (Overall: LR=1.1, *P*=0.290; 5-year LR=0.8, *P*=0.370, data not shown), a trend was observed for increased time to distant recurrence in patients with high tumor levels of TNF-α (Figure 5.5A) and high TNF-α associated with significantly improved disease-specific survival (DSS) (Figure 5.5B). When relative mRNA levels

Figure 5.4: Patients with minimal TIL infiltrate have decreased time to distant recurrence. **A)** Patients with tumors containing small to moderate numbers of CD3+ TIL display a trend for decreased dRFS (LR=4.4, *P*=0.111). This was reflected by **(B)** the CD8+ TIL subset (LR=5.5, *P*=0.063) but not **(C)** the CD4+ TIL subset (LR=2.1, *P*=0.345).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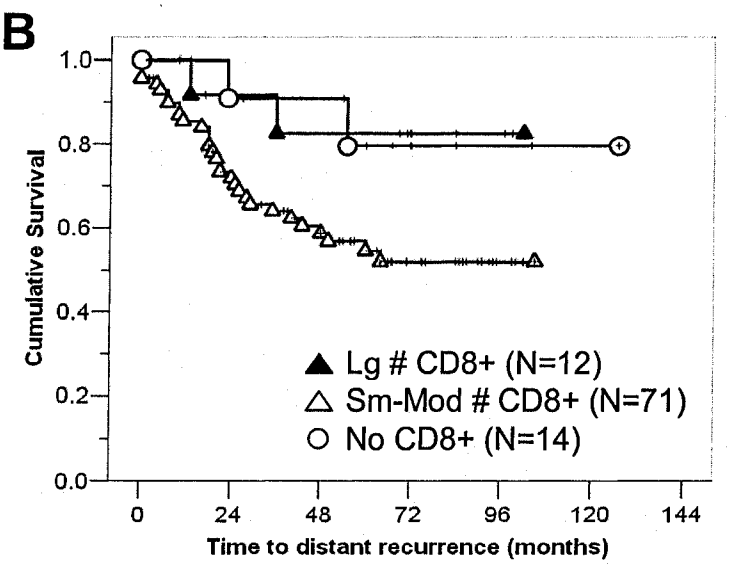
1.0
0.8
0.6
0.4
0.2
0.0
Cumulative Survival
▲ Lg # CD3+ (N=17)
△ Sm-Mod # CD3+ (N=71)
○ No CD3+ (N=11)
0
24
48
72
96
120
144
Time to distant recurrence (months)


B
1.0
0.8
0.6
0.4
0.2
0.0
Cumulative Survival
▲ Lg # CD8+ (N=12)
△ Sm-Mod # CD8+ (N=71)
○ No CD8+ (N=14)
0
24
48
72
96
120
144
Time to distant recurrence (months)

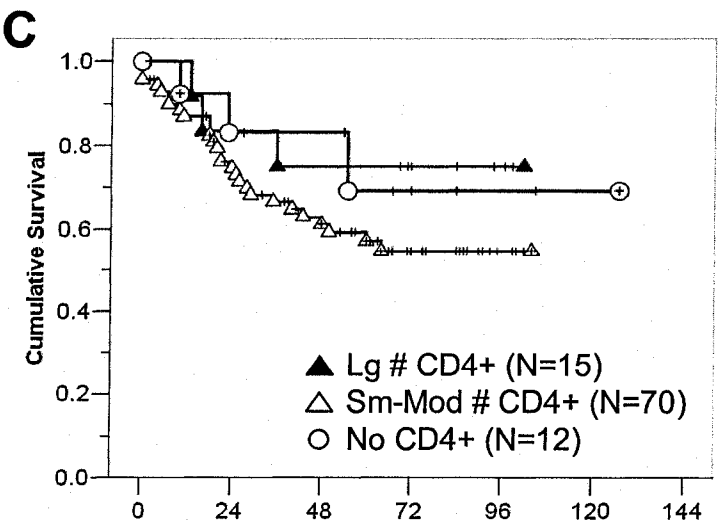

C
1.0
0.8
0.6
0.4
0.2
0.0
Cumulative Survival
▲ Lg # CD4+ (N=15)
△ Sm-Mod # CD4+ (N=70)
○ No CD4+ (N=12)
0
24
48
72
96
120
144

Table 5.4: Multivariate Cox proportional hazards models[†] to assess contribution of infiltrating CD3+ and CD8+ TIL to dRFS in breast carcinoma patients

	CD3+ TIL		CD8+ TIL		CD4+ TIL	
Variable	*P*-value	HR (95%HR)	*P*-value	HR (95%HR)	*P*-value	HR (95%HR)
TIL category:	0.031	-	0.044	-	ns[*]	-
None	0.334	2.5(0.4-15.3)	0.473	2.1(0.3-15.8)	-	
Sm-Mod#	0.012	5.0(1.4-17.4)	0.027	5.3(1.2-23.1)	-	
Lg #	*Reference*		*Reference*		*Reference*	
TNM Stage III/IV (vs I/II)	0.005	2.9(1.4-6.0)	0.005	2.9(1.4-6.1)	0.006	2.8(1.3-5.7)
PR<10fmol/mg	0.001	3.5(1.6-7.4)	0.006	2.9(1.4-6.1)	0.031	2.2(1.1-4.5)
ER< 10fmol/mg	ns	-	ns	-	ns	-
Surgery+Adjuvant vs Surgery	ns	-	ns	-	ns	-
Diagnosis Age (years)	ns	-	ns	-	ns	-
IDC (vs ILC or mixed)	ns	-	ns	-	ns	-
#events/#cases	32/91		32/89		32/89	
Model χ^2 (*P*)	20.7 (*P*<0.001)		18.9 (*P*=0.001)		11.1 (*P*=0.004)	

[†] Models employed backward stepwise method with entry at $P<0.05$ and removal at $P>0.06$.
[*] $P<0.05$, variable eliminated from the Model during backward stepwise method.

were assessed as continuous covariates in univariate Cox regression analysis, only TNF-α showed significant associations with patient survival. Similar to categorical designations, increasing TNF-α levels associated with improved DSS (P=0.041, HR(95%CI)=0.75(0.57-0.99)) and displayed a trend for improved dRFS (P=0.068, HR(95%CI)=0.82(0.66-1.02)). In multivariate survival analysis, controlling for the aforementioned prognostic parameters, high TNFα did not associate with RFS or dRFS but was an independent predictor of DSS (P=0.026, HR = 0.19; 95%CI for HR = 0.04-0.82; Model χ^2=19.4, P<0.001). Tumor stage and ER were independent predictors for RFS and dRFS, while ER expression independently predicted DSS. Similar results for DSS were obtained when TNF-α was entered as a continuous covariate (P=0.014, HR(95%CI)=0.64(0.45-0.92); Model χ^2=19.0, P<0.001)) and for 5-year survival models (data not shown).

In this sample of breast carcinoma patients we have shown that high levels of intratumoral IFN-γ (Chapter 4) [256] and TNF-α (Fig. 5.5B) associate with improved patient survival. To determine if the influence of these cytokines on patient survival is dependent on their balance with T_H2/immunoregulatory markers, ratios of ranked IFN-γ and TNF-α mRNA to IL-4, IL-10, TGF-β1 and FOX-P3 mRNA were computed, as described in Methods. Patients with a high TNF-α/TGF-β1 ratio displayed a trend for improved dRFS (Fig. 5.5C) but had significantly improved DSS (Fig. 5.5D). Survival did not significantly differ in patients stratified by other ranked mRNA ratios (data not shown). In Cox proportional hazards models controlling for the aforementioned prognostic parameters, patients with tumors in which the rank of relative TNF-α exceeded that of TGF-β1 had improved dRFS (P=0.034, HR(95%CI)=0.37(0.15-0.93);

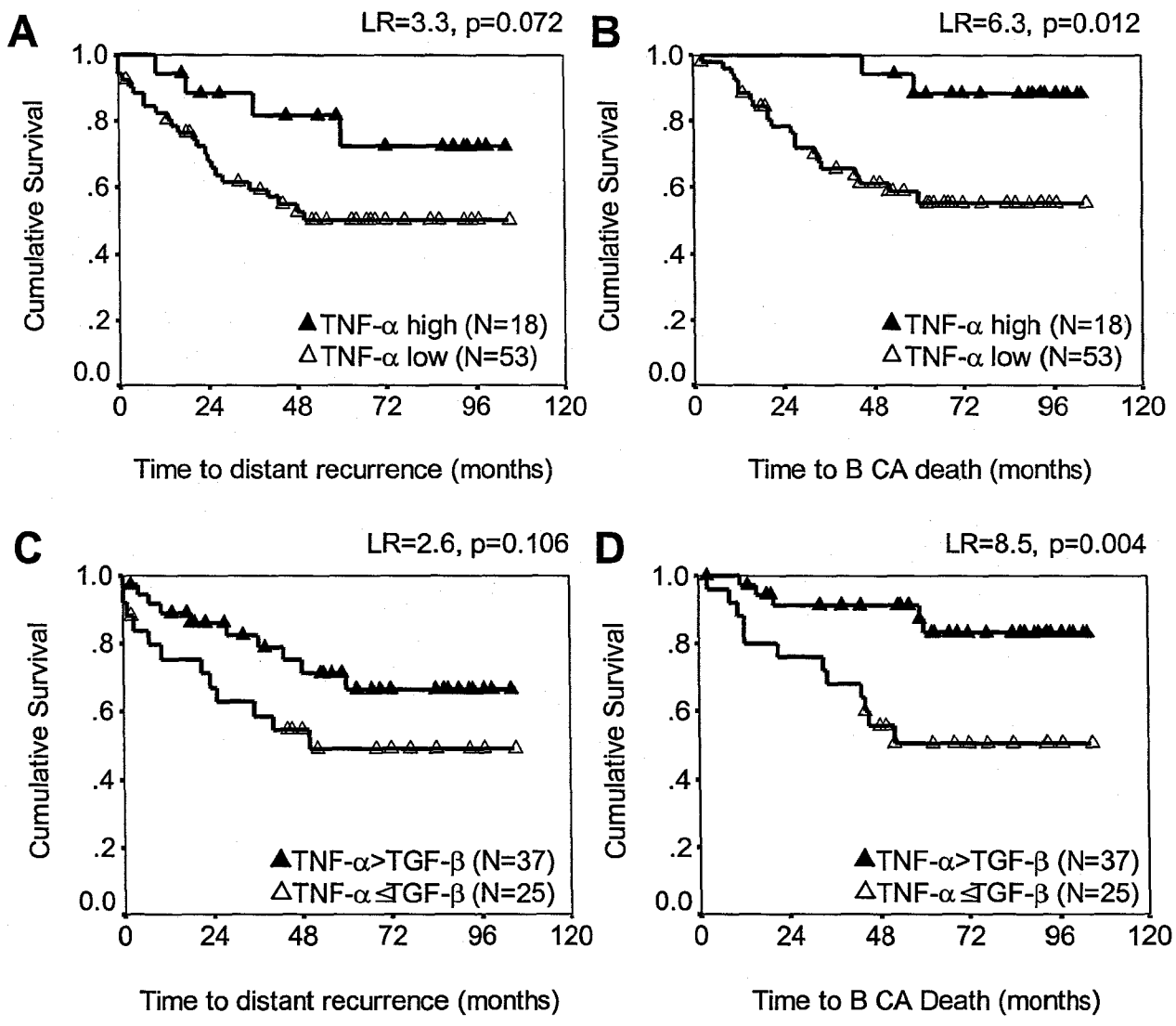
Model χ^2=19.6, P<0.001)) and DSS (P=0.001, HR(95%CI)=0.14(0.05-0.46); Model χ^2=18.7, P<0.001)).

5.5 DISCUSSION:

In support of previous immunohistochemical studies on invasive breast carcinoma, the inflammatory infiltrate was comprised predominately of CD68+ TAM and CD3+ TIL, comprised of both CD4+ and CD8+ subsets, with relatively few B lymphocytes and NK cells [144, 178]. The majority of invasive breast tumors contained an inflammatory infiltrate and a cytokine milieu comprised of both immunoregulatory and pro-inflammatory cytokines. As anticipated, positive correlations were observed between the T_H1-type cytokines IFN-γ, IL-2 and IL-12, the T_H2-type cytokines IL-4 and IL-10, and the pro-inflammatory cytokines IL-1β and IL-6. Although the findings of decreased CD8+ TIL and decreased IL-12 in recurrent lesions as compared to primary lesions are intriguing, as they may suggest decreased cellular immunity with disease progression, definitive conclusions cannot be drawn as comparative primary lesions from the same patients were not available for study. However, a small study of ovarian carcinoma patients demonstrated relative numbers of CD8+ TIL were decreased in recurrences as compared to matched primary tumors in ovarian cancer patients [278].

Variability in cytokine levels with tumor type likely reflects the distinct genetic profiles of IDC and ILC tumors as both exhibit unique differences with respect to genes involved in cell/cycle, apoptosis, angiogenesis and hormone responsiveness [279, 280]. The degree and pattern of infiltration in these tumor subtypes may also contribute to relative intratumoral cytokine mRNA levels. IDC tumors more frequently contain

Figure 5.5: Intratumoral TNF-α mRNA levels associate with improved survival in breast carcinoma patients. **A)** Patients with tumors containing high TNF-α mRNA levels display a trend for improved dRFS (5-yr % survival(95%CI) = TNF-α high: 73%(49%-96%); TNF-α low: 50%(36%-65%)). **(B)** Patients with tumors containing high TNF-α mRNA levels have improved DSS (TNF-α high: 89%(74%-100%); TNF-α low: 56%(41%-70%)).**(C)** Patients with a high intratumoral TNF-α:TGF-β1 mRNA ranked ratios have improved dRFS (TNF-α>TGF-β1: 67%(49%-84%); TNF-α ≤TGF-β1: 49(29-70)) and **(D)** DSS (TNF-α>TGF-β1: 83%(69%-97%); TNF-α ≤TGF-β1: 51(31-71)). Log rank (LR) statistic and *P*-value in upper right corners correspond to overall and 5-year comparison.


A
LR=3.3, p=0.072
Cumulative Survival
1.0
.8
.6
.4
.2
0.0
▲TNF-α high (N=18)
△TNF-α low (N=53)
0
24
48
72
96
120
Time to distant recurrence (months)
B
LR=6.3, p=0.012
Cumulative Survival
1.0
.8
.6
.4
.2
0.0
▲TNF-α high (N=18)
△TNF-α low (N=53)
0
24
48
72
96
120
Time to B CA death (months)
C
LR=2.6, p=0.106
Cumulative Survival
1.0
.8
.6
.4
.2
0.0
▲TNF-α>TGF-β (N=37)
△TNF-α≤TGF-β (N=25)
0
24
48
72
96
120
Time to distant recurrence (months)
D
LR=8.5, p=0.004
Cumulative Survival
1.0
.8
.6
.4
.2
0.0
▲TNF-α>TGF-β (N=37)
△TNF-α≤TGF-β (N=25)
0
24
48
72
96
120
Time to B CA Death (months)

diffusely distributed inflammatory infiltrates while ILC infiltrates are often perilobular [144]. We observed a similar trend as CD3+ TIL were often diffuse in IDC tumors (49/73) as compared to ILC (5/8) (Fisher's exact 2-sided test, P=0.064). Her-2/neu overexpressing tumors had significantly decreased intratumoral TGF-β1 and IL-1β. Microarray analysis of mammary tumors from MMTV-Neu transgenic mice and wild type normal mammary tissue revealed downregulation of several genes involved in the Smad-dependent TGF-β signaling cascade in Her-2/neu over-expressing tumors [281]. The negative association of Her-2/neu and IL-1β may be indirect as IL-1β positively correlated with TGF-β1 (Table 5.2) and IL-1β is known to enhance TGF-β1 production in an autocrine manner [282].

The negative associations of TIL with decreased ER levels support previous studies [174, 181, 187]. Furthermore, the association with decreased age suggests a role for circulating hormones in T cell infiltration of the tumor. Thus, in the absence of competing ligand expression on breast tumor cells, circulating estrogens may promote the recruitment and expansion of TAA-specific T cells. The hormone 17β-estradiol can modulate T cell expression of chemokine receptors and *in vitro* T cell migration [283]. Estradiol enhances the proliferation and IFN-γ production of antigen-specific CD4+ T_H1 cells *in vivo* [264] and circulating estradiol also promotes the *in vivo* expansion of CD4+CD25+FOX-P3+ regulatory CD4+ T cells [265].

Large numbers of TIL are more frequently located intratumorally and are distributed in a diffuse manner throughout the tumor mass. Large numbers of infiltrating CD4+ and CD8+ TIL associated with relative levels of T_H1 cytokines as well as the T_H2 cytokine, IL-4. Likewise, the presence of moderate to large numbers of CD20+ B cells

associated with both T_H1 and T_H2 cytokines. Indeed CD20+ B cell infiltration correlated with both CD4+ and CD8+ TIL subsets, suggesting an integration of humoral and cell mediated immunity. Following injection of murine TS/A adenocarcinoma cells into BALB/c mice, Reome et al. (2004), identified a progressive increase in CD3+ TIL with time, with substantially lower B cell infiltration which remained constant. Assessing the kinetics of infiltrating TIL subsets revealed IL-4 producing T_H2 cells emerge at the tumor at earlier time points than IFN-γ producing T_H1 cells during progressive tumor growth [284]. The failure of this integrated immune response to facilitate tumor rejection may reflect the kinetics of T cell subset infiltration but will also be dependent on the balance of anti-tumor immunity with immunoregulatory mechanisms and immune evasion strategies employed by the tumor.

The presence of T_{reg} cells within the tumor will likely suppress effector T cell responses. CD4+CD25+FOX-P3+ T_{reg} cells, capable of suppression of TAA-specific T cell immunity, are present in close proximity to CD8+ TIL in ovarian cancer and associate with advanced stage of disease and decreased survival [275]. Large numbers of $CD4^+CD25^+$ T cells with *in vitro* regulatory capacity have been isolated intratumorally and from the draining lymph nodes and peripheral blood of breast cancer patients [184]. Although T_{reg} cells were not specifically quantified in this study, we observed elevated levels of FOX-P3 mRNA in tumors that contained TIL. The positive correlation of the immunoregulatory T cell marker FOX-P3 with both T_H1 and pro-inflammatory cytokines may reflect the recruitment of T_{reg} cells to the site of inflammation. Those tumors in which the relative proportion of intratumoral TIL parallel or exceed stromal TIL contained elevated levels of T_H1-type cytokines, IL-6, IL-10 and FOX-P3, suggesting

intratumoral recruitment of T_{reg} cells. High levels of IL-6 in these tumors might represent the host response to T_{reg} mediated suppression as IL-6 induced during infection has been demonstrated to allow effector T cells to surmount T_{reg}-induced suppression [285].

Studies in ovarian carcinoma patients have also demonstrated bulk tumor FOX-P3 mRNA levels positively correlate with CD3 and IFN-γ mRNA levels [276]. Using a murine model of ovarian carcinoma, Curiel et al. (2003) showed that T_{reg} cells are recruited to the tumor in response to secretion of the chemokine CCL22 by tumor cells and intratumoral macrophages [275]. Thus the balance of effector and regulatory cell subsets will have important consequences for the induction of anti-tumor immunity and prognosis in breast carcinoma patients. Indeed, a high intratumoral CD8+/CD25+FOX-P3+ T_{reg} TIL ratio associates with improved survival in ovarian carcinoma [195]. Although we did not directly phenotypically quantify TIL subsets, the subset of patients with moderate inflammatory infiltration of their tumors showed decreased time to distant recurrence (Table 4). Tumors from these patients exhibit elevated FOX-P3 mRNA and decreased T_H1-type cytokines (Figure 2). In contrast, tumors with large numbers of TIL exhibit comparable FOX-P3 mRNA levels but showed a marked increase in levels of T_H1-type cytokines. Thus the improved survival of this subset of patients, despite the association with poor prognostic indicators, may reflect increased accumulation of effector T_H1 cells and enhanced cell-mediated immunity. In support of this, at the time of surgery, T_H1-type cytokines are elevated in tumors of small diameter (Table 3) and tumors with large numbers of CD8+ TIL have improved dRFS (Table 4). The lack of association of CD4+ TIL with patient outcome likely reflects the presence of both effector T_H cells as well as T_{reg}.

Elevated levels of the pro-inflammatory cytokines IFN-γ and TNF-α in histological grade III tumors reflect the relationship with inflammatory infiltration. Despite the association with more aggressive breast carcinoma, elevated CD8+ TIL (Table 4), TNF-α (Figure 5.5) and IFN-γ (Chapter 4) [256] associate with improved patient survival. A small study of 25 breast carcinoma patients with histological grade III tumors, demonstrated patients that experienced recurrence and metastasis (N=15) contained decreased numbers of TNF-α expressing inflammatory cells, which associated with lower numbers of apoptotic cancer cells, and higher tumor proliferation rates as compared to histopathologically matched patients who remained recurrence-free for 5-years [286]. In a murine model of breast carcinoma, microsphere delivery of IL-12 and TNF-α into established tumors associated with increased CD4+ and CD8+ TIL infiltration, production of IFN-γ and tumor regression and provided immunological memory capable of tumor rejection upon rechallenge [287].

TNF-α is a pleiotropic growth factor secreted mainly by T cells and macrophages that has a paradoxical role in carcinoma. Experimental models of carcinogenesis have demonstrated low level TNF-α production may foster a chronic inflammatory milieu, leading to increased angiogenesis, production of matrix degrading enzymes and tumor metastasis, while high intratumoral levels of TNF-α lead to cancer destruction via direct growth inhibitory effects on tumors cells and/or indirect action on tumor infiltrating cells, leading to tumor necrosis and apoptosis [288]. An additional role for high concentrations of TNF-α may be via its activity on immunosuppressive T_{reg} cells in the tumor microenvironment. Recently, high concentrations of TNF-α were found to inhibit the *in vitro* immunoregulatory effects of both naturally occurring and TGF-β1 induced

CD4+CD25+ T_{reg} of healthy individuals, via signaling through TNFRII expressed on T_{reg}, which correlated with downregulated FOX-P3 expression [289]. In support of this hypothesis, survival was markedly increased in patients whose tumors display elevated ranked TNF-α/TGF-β1 ratios (Figure 5.5D).

The ability of high levels of tumoral TNF-α to independently predict improved disease specific survival may also reflect the enhanced delivery of chemotherapeutic regimens, to areas of residual disease, via TNF-α mediated induction of increased vascular permeability [290]. In support of this, high TNF-α associated with improved survival (LR=5.7, P=0.017) in the subset of patients treated with adjuvant chemotherapy (N=33, data not shown).

In conclusion, the results of this study provide further insight into the complexity of the immune cell infiltrate in breast carcinoma. Much work remains to be done to understand the unique properties of tumor infiltrating inflammatory cells and how their cytokine responses are regulated in the context of the tumor microenvironment. A thorough understanding of the anti-tumor immune response will aid in the development of immunotherapeutic regimes in breast carcinoma.

Supplementary Table 5S1: Primer sequences for detection of mRNA in breast carcinoma lesions.

	Sense Primer (5'-3')	Anti-sense Primer (5'-3')	Size (bp)	GenBank Accession Number*
β-Actin	ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG	CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC	840	NM_005159
CD3δ	CTG GAC CTG GGA AAA CGC ATC	GTA CTG AGC ATC ATC TCG ATC	311	NM_000732
IFN-γ	AGT TAT ATC TTG GCT TTT CA	ACC GAA TAA TTA GTC AGC TT	356	J00219
IL-2	ACT CAC CAG GAT GCT CAC AT	AGG TAA TCC ATC TGT TCA GA	269	HSU25676
IL-12 p40	CCA AGA ACT TGC AGC TGA AG	TGG GTC TAT TCC GTT GTG TC	355	NM_002187
IL-4	CCT CTG TTC TTC CTG CTA GCA TGT GCC	CCA ACG TAC TCT GGT TGG CTT CCT TCA	371	M23442
IL-10	ATG CCC CAA GCT GAG AAC CAA GAC CCA	TCT CAA GGG GCT GGG TCA GCT ATC CCA	352	NM_000572
TGF-β	GCC CTG GAC ACC AAC TAT TGC	AGG CTC CAA ATG TAG GGG CAG G	165	X02812.1
FOX-P3	CAG CTG CCC ACA CTG CCC CTA G	CAT TTG CCA GCA GTG GGT AG	384	AF277993
IL-1β	ACA GAT GAA GTG CTC CTT CCA	GTC GGA GAT TCG TAG CTG GAT	75	BT007213
IL-6	AGC TCA GCT ATG AAC TCC TTC TC	GTC TCC TCA TTG AAT CCA GAT TGG	340	NM_000600
TNF-α	CGG GAC GTG GAG CTG GCC GAG GAG	CAC CAG CTG GTT ATC TCT CAG CTC	354	NM_000594

* All primer sequences were validated using published GenBank mRNA sequences.

Supplementary Table 5S2: Infiltrating cell subsets[†] in primary and recurrent breast tumors

		Primary Tumors		Recurrent Tumors		*P*-value[*]
		N	(%)	N	(%)	
CD3+	None	11	(11.1)	4	(26.7)	0.352
	Sm#	30	(30.3)	5	(33.3)	
	Mod#	41	(41.4)	4	(26.7)	
	Lg#	17	(17.2)	2	(13.3)	
CD4+	None	15	(15.5)	4	(28.6)	0.646
	Sm#	36	(37.1)	4	(28.6)	
	Mod#	34	(35.1)	4	(28.6)	
	Lg#	12	(12.4)	2	(14.3)	
CD8+	None	14	(14.4)	6	(42.9)	**0.040**
	Sm#	41	(42.3)	2	(14.3)	
	Mod#	30	(30.9)	5	(35.7)	
	Lg#	12	(12.4)	1	(7.1)	
CD20+	None	77	(78.6)	12	(80.0)	0.982
	Sm#	14	(14.3)	2	(13.3)	
	Mod#	6	(6.1)	1	(6.7)	
	Lg#	1	(1.0)	0	(0)	
CD56+	None	41	(95.3)	7	(87.5)	0.366
	Sm#	1	(2.3)	1	(12.5)	
	Mod#	1	(2.3)	0	(0)	
	Lg#	0	(0)	0	(0)	
CD68+	None	0	(0)	0	(0)	0.165
	Sm#	8	(8.1)	3	(20.0)	
	Mod#	32	(32.3)	2	(13.3)	
	Lg#	59	(59.6)	10	(66.7)	

[†]Of the 99 primary and 15 recurrent tumors evaluated for immune cell infiltration, all had information on $CD3^+$ and $CD68^+$ cells. Due to lack of tissue availability or tissue loss during staining procedure, information on infiltrating CD20+, CD4+, CD8+ and CD56+ cells was available for 98, 97, 97, and 43 primary tumors and 15, 14, 14 and 8 recurrent tumors, respectively.

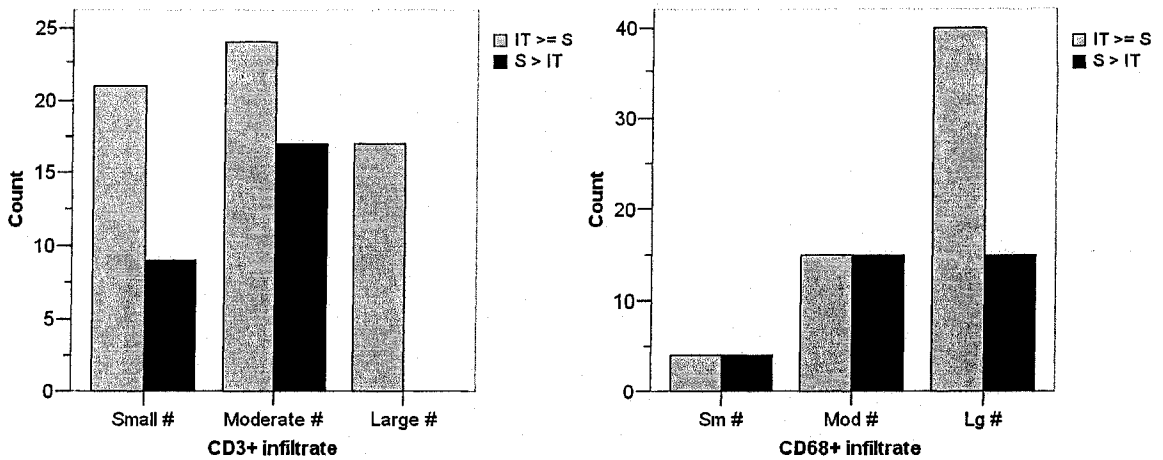
[*]*P*-value calculated using Pearson's Chi-square test.

Supplementary Table 5S3: Relative cytokine and FOX-P3 mRNA levels in primary and recurrent breast tumors

		Primary Tumors				Recurrent Tumors				*P*-value*
	mRNA	(N)	Mean±SEM	Min-Max	% negative†	(N)	Mean±SEM	Min-Max	% negative†	
T cell	CD3	(81)	33.8±2.3	0-82.3	1.2	(9)	29.9±7.6	1.9-67.0	0	0.619
T_H1/cellular immunity	IFN-γ	(82)	10±1.5	0-71.3	18.3	(9)	11.0±4.0	0-32.0	22.2	0.779
	IL-2	(81)	11.9±1.2	0-46.5	3.7	(9)	8.8±3.6	0-35.3	11.1	0.404
	IL-12	(75)	7.2±0.8	0-35.7	8.0	(8)	2.6±1.2	0-9.8	37.5	**0.030**
$T_H2/T_H3/T_{reg}$	IL-4	(76)	1.2±0.3	0-17.4	51.3	(8)	0.3±0.3	0-2.1	75.0	0.174
	IL-10	(75)	22.1±1.5	0-48.4	1.3	(7)	17.6±4.6	3.5-41.3	0	0.374
	TGF-β	(69)	54.5±2.7	9-148.2	0	(8)	58.8±10.1	33.7-107.3	0	0.815
	FOX-P3	(77)	17.4±4.0	0-157.3	15.6	(9)	2.7±0.9	0-7.6	33.3	0.188
Pro-inflammatory	IL-1β	(77)	32.6±4.1	3-280	0	(9)	14.9±2.7	0-24.1	11.1	0.073
	IL-6	(77)	34.1±3.1	0-125.3	2.6	(9)	19.9±4.9	0-44.1	11.1	0.164
	TNF-α	(71)	1.8±0.3	0-12.8	32.4	(9)	0.7±0.3	0-2.3	55.6	0.137

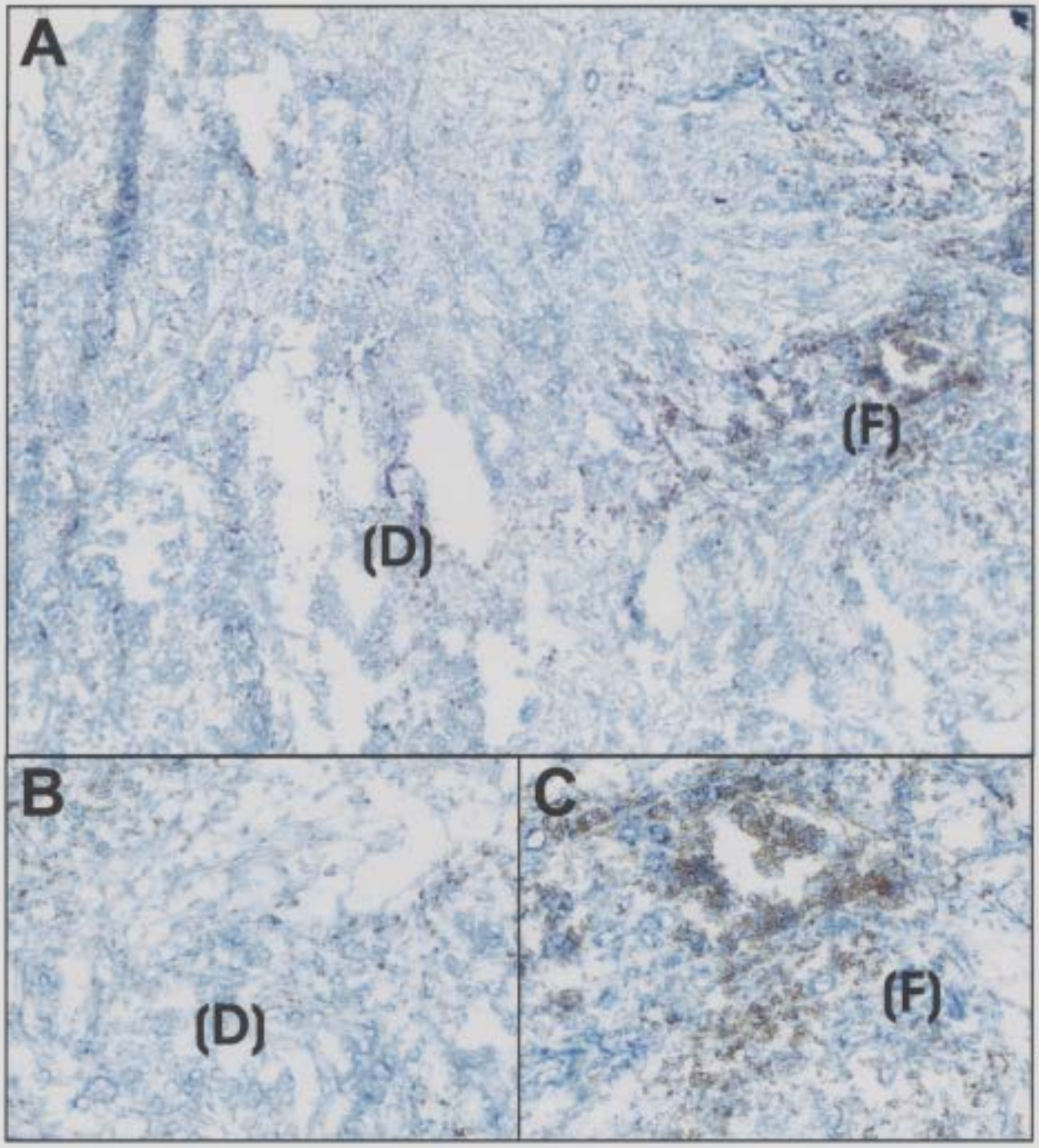
*_P_-value calculated using Mann-Whitney U test.

†Percentage of tumors with undetectable mRNA (Relative mRNA spot density ratio to β-actin=0).

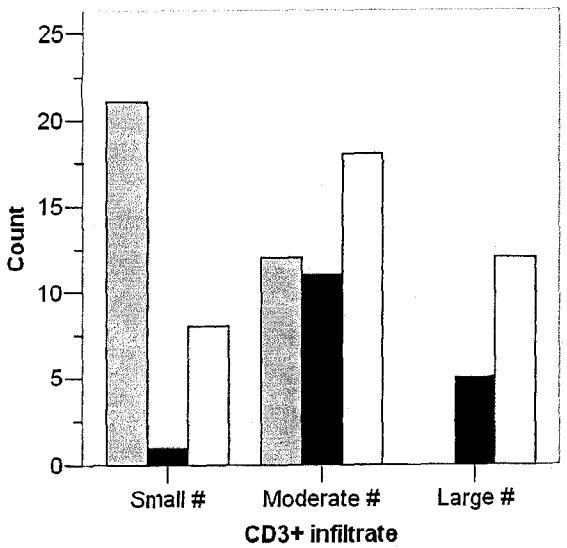


Supplementary Figure 5S1: Large numbers of CD3+ TIL and CD68+ TAM associate with intratumoral location (CD3: χ^2=9.9, *P*=0.007; CD68: χ^2=5.0, *P*=0.082). IT=intratumoral, S=stromal, TIL=tumor infiltrating lymphocytes, TAM = tumor associated macrophages.

Supplementary Figure 5S2: Representative immunohistochemistry example illustrating focal and diffuse distribution of infiltrating CD3+ cells in breast carcinoma. **(A)** In this representative example, moderate numbers of infiltrating CD3+ cells (mAb UCHT1) were detected both diffusely distributed (D) throughout tumor stroma **(B)** and in focal (F) aggregates **(C)**. Original magnifications of 40X (A) and 200X (B-C).


A
(D)
(F)
B
(D)
C
(F)

Supplementary Figure 5S3: Distribution of infiltrating cell populations in primary breast carcinoma lesions. Small numbers of CD3+, CD4+ and CD8+ TIL were primarily focally aggregated in the tumor mass, while large numbers were more frequently diffusely distributed throughout the tumor. CD20+ B cells were relatively infrequent and showed a predominantly focal distribution. CD68+ TAM were diffusely distributed throughout the tumor mass. F=focal aggregation of infiltrating cells, D=diffuse distribution of infiltrating cells, F+D=focal aggregates and diffusely distributed cells.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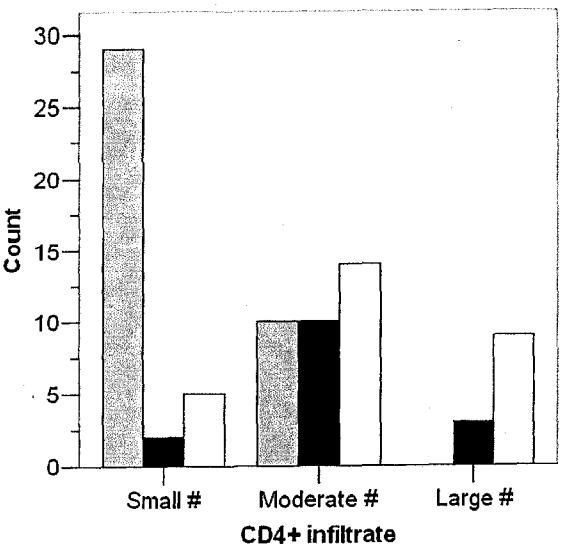
20
15
10
5
0
Small #
Moderate #
Large #
CD3+ infiltrate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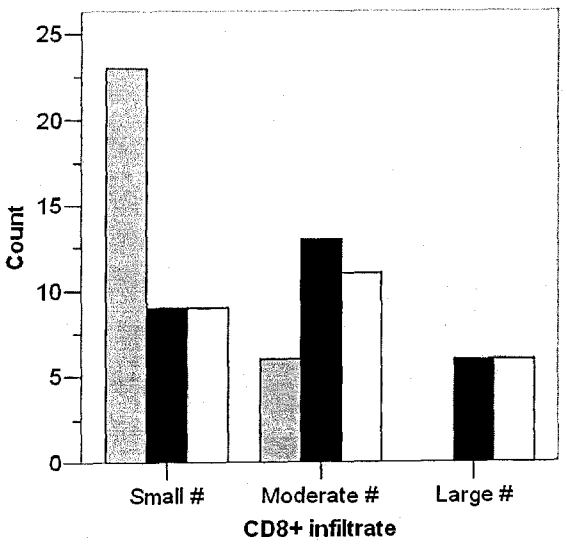
25
20
15
10
5
0
Small #
Moderate #
Large #
CD4+ infiltrate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20
15
10
5
0
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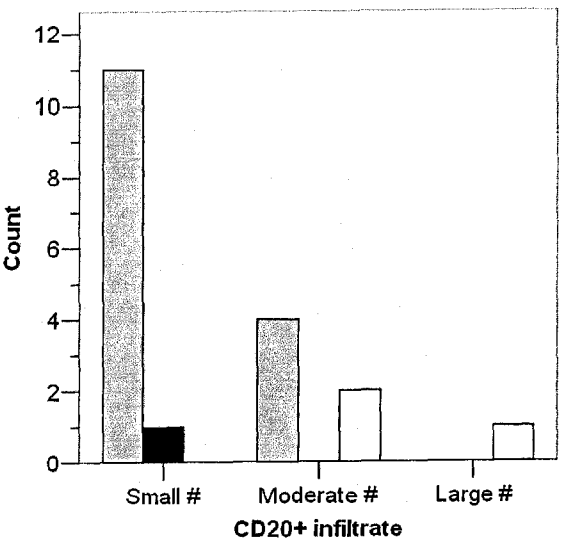
Moderate #
Large #
CD8+ infiltrate


Count
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10
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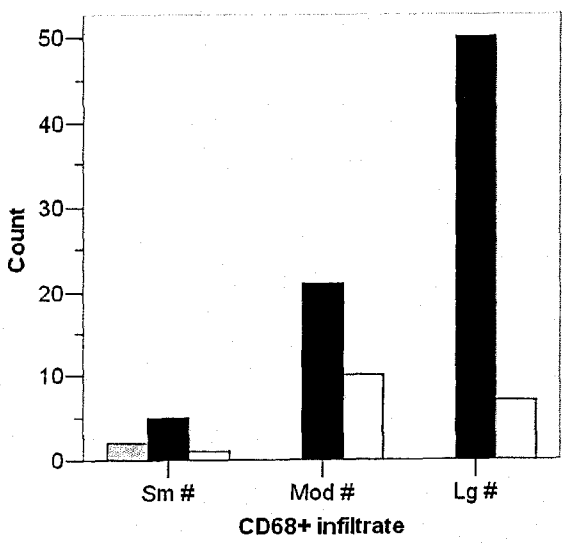
6
4
2
0
Small #
Moderate #
Large #
CD20+ infiltrate


Count
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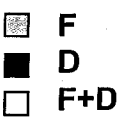
30
20
10
0
Sm #
Mod #
Lg #
CD68+ infiltrate


F
D
F+D

CHAPTER 6: RELATIONSHIP OF SINGLE NUCLEOTIDE POLYMORPHISMS IN CYTOKINE GENES WITH INTRATUMORAL CYTOKINE LEVELS AND PROGNOSIS IN BREAST CARCINOMA[†]

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[†] Manuscript in preparation.

6.1 ABSTRACT

The anti-tumor immune response is dependent on the intratumoral cytokine milieu, which regulates the function of tumor infiltrating immune effector cells. Cytokine genes display a high degree of polymorphism and allelic variation associates with variability in cytokine levels. Studies investigating associations of cytokine gene polymorphisms with disease susceptibility and prognosis in breast carcinoma have yielded conflicting results. To determine if associations of cytokine gene single nucleotide polymorphisms (SNPs) with prognostic parameters are attributable to variation in the levels of intratumoral cytokine production, we assessed the relationships of cytokine SNPs with prognostic parameters and relative intratumoral cytokine mRNA levels in a pilot study of 60 invasive breast carcinoma patients. Significant associations were observed between the IL-1β -511+3962 TC haplotype and tumor grade and decreased patient survival, the IL-6 -174nt565 CA haplotype and elevated tumor ER levels and the IL-10 -1082-819-592 GCC haplotype with decreased ER and PR. These associations were independent of variability in their respective cytokine mRNA levels, suggesting the prognostic influence of these allelic variants is attributable to unidentified linked genetic variants and/or additional factors that control cytokine production in the tumor microenvironment. Relative levels of IL-2, and IFN-γ demonstrated significant positive associations with the IL-2 -330+166 TG haplotype and the IFN-γ +874 T allele, respectively. However, assessment of these relationships in tumors subgrouped by proportions of CD3+ TIL suggest genetic variation in cytokine genes play a minor role in intratumoral cytokine production in the presence of large numbers of TIL. The results of this exploratory study suggest the true prognostic significance of genetic variation in cytokine genes in breast

carcinoma is presently unclear and will require extensive study of large numbers of patients with extended haplotype analysis. Furthermore, the interpretation of such studies will require a thorough knowledge of the full contribution of extended SNP haplotypes on cytokine production in response to various stimuli and in different cell types.

6.2 INTRODUCTION

Cytokine genes are highly conserved in terms of their exon sequences, however, polymorphisms within the regulatory region of cytokine genes can influence transcription via alteration of transcription factor binding, while intronic polymorphisms may influence mRNA splicing or the structure of gene enhancer or silencer regions [291]. Breast carcinoma lesions frequently contain an inflammatory cell infiltrate and there is substantial evidence to support a role for immune responsiveness in disease development and progression. The anti-tumor immune response is controlled by the balance of cytokines secreted by infiltrating immune cells and/or tumor cells. This regulation is accomplished by the ability of different cytokines to act synergistically or antagonistically. For example, IFN-γ enhances TNF-α mediated tumor cell growth inhibition and apoptosis, but acts antagonistically with IL-4 in the production of IgG subclasses by B lymphocytes [292].

Family and twin studies have demonstrated a strong genetic influence on cytokine secretion from *in* vitro stimulated human peripheral blood mononuclear cells (PBMC) [293], suggesting a genetic basis for control of cytokine production. As single nucleotide polymorphisms (SNPs) of cytokine genes can influence cytokine gene promoter activity [201, 204] and associate with variable levels of cytokine production, following *in vitro* cell stimulation or *in vivo* in the plasma of healthy donors [204, 205], several studies have

attempted to correlate the carriage of such allelic variants with disease susceptibility and prognosis in breast carcinoma. However, in both instances discrepant reports have arisen [214, 218, 219].

The discrepancies of such reports may in part reflect ethnic differences in allele distribution; however, such studies have failed to examine the associations of SNPs with prognosis in the context of intratumoral cytokine levels. In an attempt to investigate whether the associations of SNPs of cytokine genes with prognostic parameters are attributable to an influence on immune responsiveness, we have molecularly typed 60 invasive breast carcinoma patients for SNPs of cytokine genes and evaluated their relationship to prognostic parameters and relative intratumoral cytokine mRNA levels.

6.3 MATERIAL AND METHODS

6.3.1 Tumor samples and patient characteristics

Tumors were randomly selected, based on tissue availability, from a larger sample with prognostic and outcome information, obtained from the Manitoba Breast Tumor Bank (MBTB) (Winnipeg, Manitoba, Canada), with approval of the local Human Investigation Committees. The median age of initial diagnosis was 61 years (mean±sd = 59.4±14.0 years). The median follow-up time was 61 months (mean±sd = 70.9±56.2 months; range 2-215 months), during which 19 patients died of breast cancer. The median time to recurrence was 46 months (mean±sd = 53.3±44.3 months; range 2-328), during which 31 patients experienced recurrences (20 distant, 7 regional and 4 distant+regional). Breast tumor samples were comprised of 52 primary tumor lesions and 8 recurrent tumors. Primary tumor prognostic information was available for 59 patients for tumor type (infiltrating ductal carcinoma (IDC): n=52, infiltrating lobular carcinoma

(ILC): n=7) and lymph node status (LN-: n=26; LN+: n=33). Primary tumor size was available for 57 patients (≤2 cm: n=13; >2cm: n=44). Fifty-five patients were staged according to AJCC guidelines [8] for TNM stage (Stage I/II: n=43; Stage III/IV: n=12). Primary tumor estrogen receptor (ER) and progesterone receptor (PR) levels were determined by ligand binding assay and available for 52 patients (ER: median = 16.5 fmol/mg, mean±sd = 47.9±68.3 fmol/mg; PR: median = 16.6 fmol/mg, mean±sd = 71.3±163.9 fmol/mg). Primary tumors were graded for 49 patients (Grade I: n=3; Grade II: n=28; Grade III: n=18), determined using the Nottingham grading system [6]

6.3.2 DNA extraction and cytokine SNP genotyping

DNA was isolated from thawed, fresh-frozen breast tumor tissues by homogenization using the DNAzol reagent (Gibco BRL, Rockville, MD). Single nucleotide polymorphisms (SNPs) in cytokine genes were detected by PCR with sequence-specific primers, using Pel-Freez Cytokine genotyping kits (Pel-Freez Clinical Systems, LLC®, Brown Deer, WI, USA). DNA from breast tumors was typed for SNPs in IL-1β (-511C>T, +3962T>C), IL-1R (pst1 1970C>T), IL-1RN (mspa1 11100T>C), IL-2 (-330T>G, +166G>T), IL-4 (-1098T>G, -590T>C, -33T>C), IL-6 (-174G>C, nt565G>A), IL-10 (-1082G>A, -819C>T, -592C>A), IL-12 (-1188C>A), IFN-γ (+874A>T), TGF-β1 (Codon10C>T, Codon25G>C), TNF-α (-308G>A, -238G>A). PCR reactions were carried out using a Biometra T Gradient thermocycler (Montreal Biotech Inc., Quebec, Canada) following manufacturer's instructions (Pel-Freez Clinical Systems) with the addition of a final 5-minute extension step at 72°C at the end of thermal cycling.

6.3.3 RNA extraction and semi-quantitative RT-PCR

Total RNA was isolated from breast tumor tissues by homogenization using the Trizol reagent (Gibco BRL, Rockville, MD), followed by treatment with DNA-free reagent (Ambion, Austin, TX) to remove any contaminating DNA. Reverse transcription was performed on 1 μg RNA using the First Strand cDNA Synthesis Kit (Pharmacia Biotech, Quebec, Canada). PCR was performed using a Biometra T Gradient thermocycler (Montreal Biotech Inc., Quebec, Canada) to amplify cDNA using primers described in Table 6.1. All PCR reactions were performed in a volume of 50 μL with 200 μM dNTPs (Gibco BRL) and 1 μL cDNA. PCR buffer contained 20 mM Tris-HCl (pH 8.4) and 50 mM KCl. Samples containing water instead of test cDNA were included as contamination controls and cDNA from the cell lines Jurkat E6-1 (IL-2, IL-4, IL-10, TGF-β), C10/MJ (IFN-γ, IL-6), U937 (TNF-α, IL-1β) and YAR (IL-12) were used as positive controls. Primers were synthesized by Gibco BRL and included β-actin [251], IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-12 p40, TNF-α [252], IL-1β [253], and TGF-β1 [254]. Primers were used at concentrations of 20 pM for β-actin, IL-1β, IL-6, and TNF-α and 10pM for all others. MgCl2 (Gibco BRL) concentration was 1.5 mM for β-actin, and IL-4 and 2 mM for all other reactions. 0.2 μL of Taq DNA polymerase (Gibco BRL) was used for β-actin, IL-1β, and IL-6 reactions and all other reactions used 0.25 μL. Reaction mixtures were amplified for 35 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 55°C (IFN-γ and IL-2), 72°C (TNF-α) or 65°C (all others), and extension at 72°C for 1 min, followed by a separate 5 min extension step at 72°C for all but IL-1β, and IL-6. The intensity of amplified products was semi-quantified and normalized as a percent of β-actin using the ChemiImager 4000 with Alphaease 4.0 Software.

Table 6.1: Primer sequences for detection of cytokine mRNA in breast carcinoma lesions.

	Sense Primer (5'-3')	Anti-sense Primer (5'-3')	Size (bp)	GenBank Accession Number*
β-Actin	ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG	CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC	840	NM_005159
IFN-γ	AGT TAT ATC TTG GCT TTT CA	ACC GAA TAA TTA GTC AGC TT	356	J00219
IL-2	ACT CAC CAG GAT GCT CAC AT	AGG TAA TCC ATC TGT TCA GA	269	HSU25676
IL-12 p40	CCA AGA ACT TGC AGC TGA AG	TGG GTC TAT TCC GTT GTG TC	355	NM_002187
IL-4	CCT CTG TTC TTC CTG CTA GCA TGT GCC	CCA ACG TAC TCT GGT TGG CTT CCT TCA	371	M23442
IL-10	ATG CCC CAA GCT GAG AAC CAA GAC CCA	TCT CAA GGG GCT GGG TCA GCT ATC CCA	352	NM_000572
TGF-β	GCC CTG GAC ACC AAC TAT TGC	AGG CTC CAA ATG TAG GGG CAG G	165	X02812.1
IL-1β	ACA GAT GAA GTG CTC CTT CCA	GTC GGA GAT TCG TAG CTG GAT	75	BT007213
IL-6	AGC TCA GCT ATG AAC TCC TTC TC	GTC TCC TCA TTG AAT CCA GAT TGG	340	NM_000600
TNF-α	CGG GAC GTG GAG CTG GCC GAG GAG	CAC CAG CTG GTT ATC TCT CAG CTC	354	NM_000594

* All primer sequences were validated using published GenBank mRNA sequences.

6.3.4 Immunohistochemistry

Commercially available monoclonal antibodies (mAb) (BD Biosciences Pharmingen, Mississauga, ON, Canada) were used to assess infiltrating $CD3^+$ TIL (clone UCHT1, 2.5 μg/ml, Pharmingen) and identify tumor cells (anti-cytokeratin mAb clone AE1/AE3, 5 μg/ml, DakoCytomation) Isotype matched non-specific mouse immunoglobulins (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) served as negative controls. Serial frozen sections (8μm) were fixed in acetone for 10 minutes at –20°C, shipped from the MBTB and stored at –70°C until immunohistochemical staining. After thawing, drying and rehydrating in phosphate buffered saline (PBS) (pH 7.4), sections were treated with 1.5% hydrogen peroxide in PBS for 30 minutes to remove endogenous peroxidases, and nonspecific binding was blocked with 15% goat serum in PBS for 1 hour. Sections were incubated for 1 hour with primary antibody followed by incubation for 30 minutes with goat anti-mouse DAKO EnVision horseradish peroxidase labeled polymer (DAKO Diagnostics Canada Inc., Mississauga, ON, Canada). Antibody binding was visualized by incubating with diaminobenzidine + hydrogen peroxide (Sigma, Oakville, ON, Canada) for 5 minutes. The reaction was stopped with water and sections were counterstained in Mayer's hematoxylin. Hematoxylin and eosin staining was performed on one section for each breast carcinoma and samples where tumor cells comprised <10% of the section area were excluded from analysis. All slides were coded and independently examined by two readers, in the absence of information on prognostic parameters.

Infiltrating CD3+ TIL were coded based on estimated numbers: - (no or a few scattered cells); -/+ (small numbers of scattered cells or occasional small aggregates); +

(moderate numbers of scattered cells, numerous small aggregates or occasional large aggregates); ++ (large numbers of scattered cells or several large aggregates).

6.3.5 Statistical Analysis

Contingency tables were analyzed using Pearson's chi-square analysis or Fisher's exact two-sided t-test for 2x2 tables with expected counts ≤ 5. Non-parametric Mann-Whitney (2 category variables) and Kruskal-Wallis (≥2 category variables) were used when assessing statistical significance of continuous variables. All cytokine SNP genotypes were tested to ensure their frequencies were in agreement with those expected from allele frequencies using the Hardy-Weinberg equation ($1=p^2+2pq+q^2$), with deviations from expected assessed using χ^2-tests. *P*-values were corrected (*Pc*) for multiple comparisons using the Bonferroni method according to the formula $Pc = 1 - (1 - P)^n$, where *Pc* is the corrected value, *P* is the uncorrected value, and n is the number of comparison groups.

Survival estimates were calculated using Kaplan-Meier method with log-rank statistic. Ninety-five percent confidence intervals (95% CI) around 5-year percent survival estimates were calculated using the standard error (SE) of the cumulative survival probability (95%CI = cumulative survival probability ±1.96 x SE). Estimates were calculated as time to regional or distant metastasis for recurrence-free survival (RFS), time to distant metastasis for distant recurrence-free survival (dRFS) and time to death from breast cancer for disease-specific survival (DSS). For disease-free survival, patients who died of other causes or who were alive with other malignancy were censored from analysis. Backward stepwise Cox proportional hazards models were constructed for multivariate survival analyses, using a probability of $P<0.05$ for stepwise entry and

$P>0.06$ for removal. No patients were lost to follow-up and patients not experiencing the event were censored at the time of last follow-up or at 5 years for 5-year survival. For multivariate analysis, correlation matrices were constructed to ensure lack of collinearity of covariates. For all statistical tests, differences between groups were considered significant if $P<0.05$. All analysis was performed using SPSS Version 11.5 statistical software.

6.4 RESULTS AND DISCUSSION

To determine if levels of intratumoral cytokine mRNA relate to associations of genetic polymorphisms of cytokine genes and prognostic parameters in breast carcinoma, 60 invasive breast tumors were DNA-typed for single nucleotide polymorphisms (SNPs) of their cytokine genes and evaluated for relative intratumoral mRNA levels using RT-PCR. All SNP genotypes followed Hardy Weinberg equilibrium (see Appendix III), except the IL-4 -1098 genotype ($\chi^2=7.6$, $P=0.023$), which was excluded from analysis. With the exception of the TNF-α -308-238 AG haplotype, which was increased in recurrent tumors (6/8 (75%) recurrent versus 16/52 (30.8%) primary tumors, Fisher's exact test $P=0.042$), cytokine SNPs did not differ between primary and recurrent tumors. Furthermore, associations of cytokine SNP alleles/genotypes with relative cytokine mRNA levels were similar in the 52 primary tumors and in the combined primary and recurrent tumor set (see Appendix III). Therefore significant associations are depicted for the combined tumor set. Allelic variation in IL-4, IL-10, IL-12 and TNF-α genes did not associate with prognostic parameters, patient survival or intratumoral cytokine mRNA levels. In contrast SNPs in IL-1β, IL-6, IL-10, TGF-β1, IL-2 and IFN-γ showed

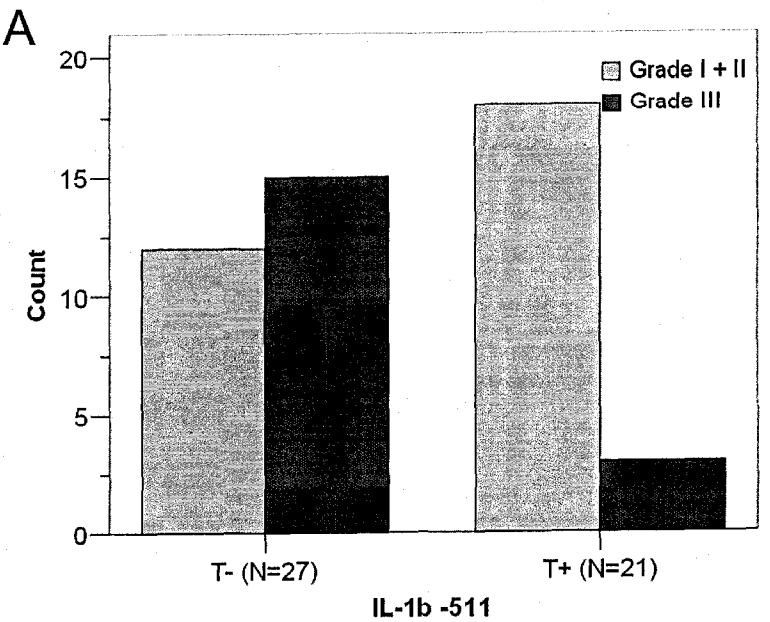
associations with clinicopathological parameters and/or intratumoral cytokine mRNA levels, as discussed in the following sections.

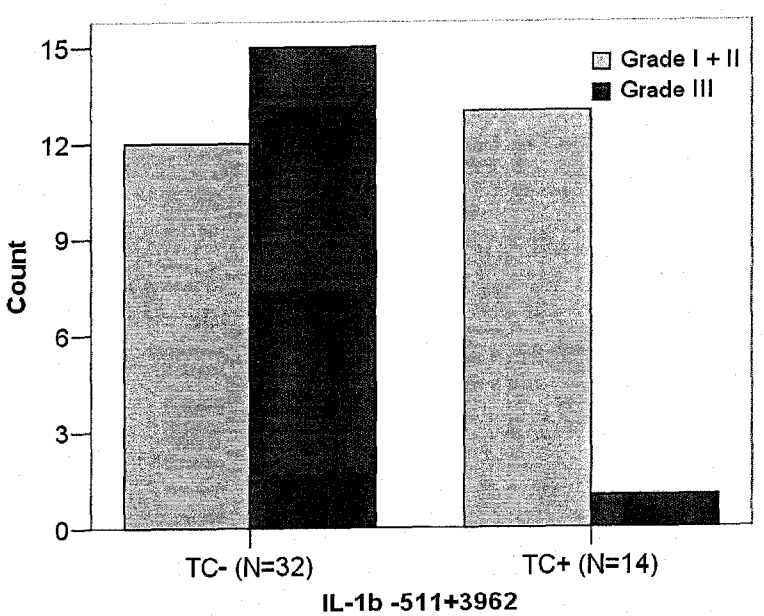
6.4.1 IL-1β

Members of the IL-1 family of cytokines and receptors are expressed in tumor and stromal cells of breast carcinoma tissue and correlate with poor prognostic indicators and pro-angiogenic markers [294]. IL-1β represents a prototypical pleiotropic pro-inflammatory cytokine and within the tumor environment, elevated IL-1β can promote tumor growth, invasion and metastasis [257]. We examined two polymorphisms of the IL-1β gene; the -511 C->T transition of the IL-1β promoter and the +3962 T>C transition located in exon 5 of the coding sequence.

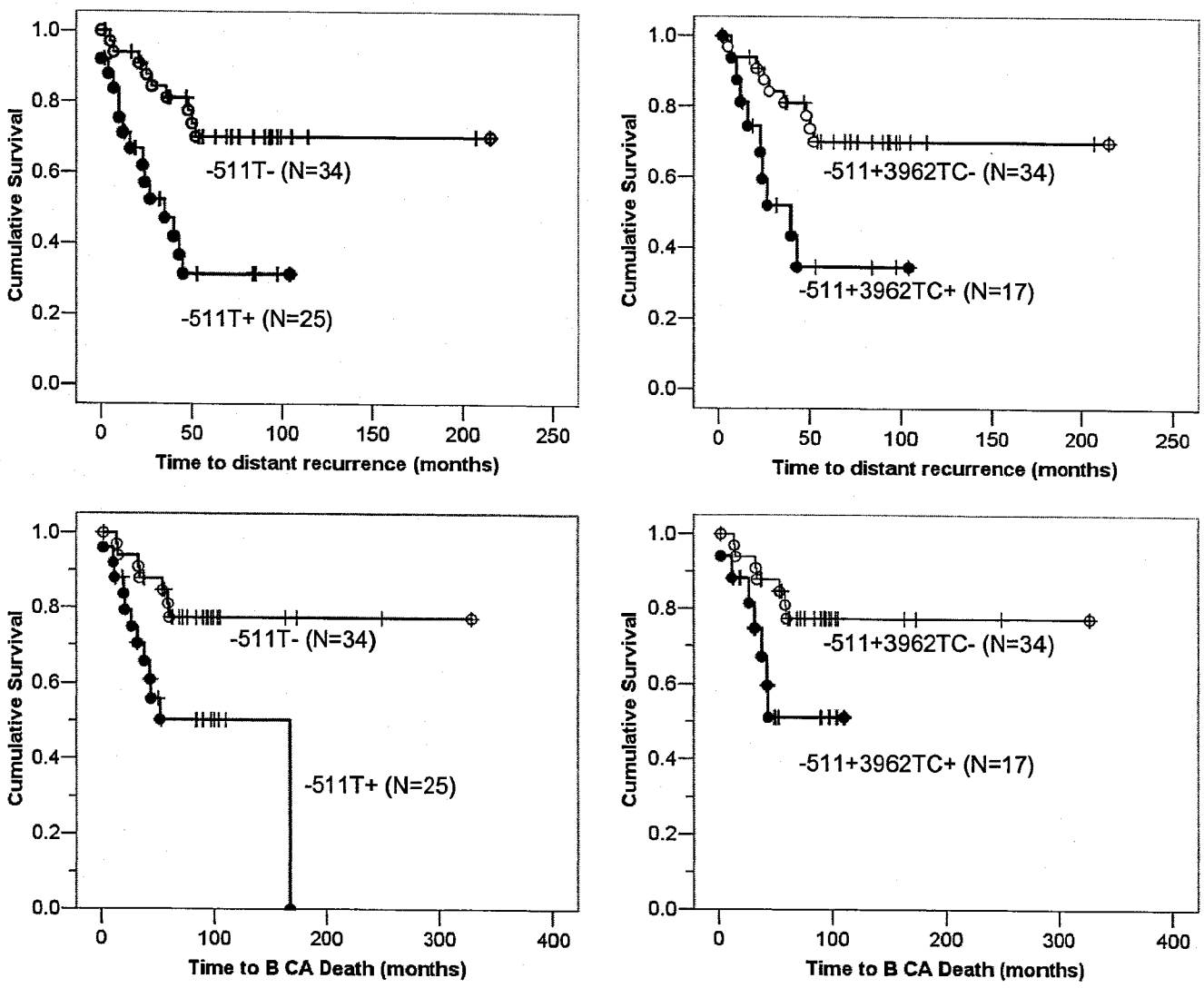
Stratification of breast carcinoma patients by clinicopathological parameters revealed the IL-1β -511T allelic variant associated with lower tumor grade, especially in individuals that carry the -511+3962 TC haplotype (Figure 6.1A). Despite the association with well-differentiated tumors, the IL-1β -511T allelic variant associates with decreased recurrence-free survival (RFS: LR=8.4, P=0.004, data not shown), distant recurrence-free survival (Figure 6.1B) and disease-specific survival (Figure 6.1B). This was particularly evident for individuals that carry the IL-1β -511+3962 TC haplotype (RFS: LR=5.8, P=0.017, data not shown; dRFS, DSS: Figure 1C). The IL-1β -511+3962 TC haplotype remained an independent predictor of poor DSS in multivariate Cox regression proportional hazards analysis, controlling for tumor stage, tumor grade, diagnosis age and ER expression (P=0.016, HR (95%CI)=4.52(1.32-15.47); Model χ^2=8.9, P=0.012). The observed associations of the IL-1β -511T allele or -511+3962 TC haplotype with

Figure 6.1: Associations of IL-1β SNPs with prognostic factors and survival in invasive breast carcinoma. **A)** The IL-1β -511T allelic variant associates with decreased tumor grade (Pearson's $\chi 2=8.6$, P=0.003). This was particularly evident for individuals that carry the IL-1β -511+3962 TC haplotype (Pearson's $\chi 2=9.1$, P=0.003). **B)** The IL-1β -511T allelic variant associates with decreased distant recurrence-free survival (dRFS: LR=10.2, P=0.001) and disease-specific survival (DSS: LR=7.3, P=0.007). This was particularly evident for individuals that carry the IL-1β -511+3962 TC haplotype (dRFS: LR=6.6, P=0.010; DSS: LR=4.8, P=0.028).


A
Grade I + II
Grade III
Count
20
15
10
5
0
T- (N=27)
T+ (N=21)
IL-1b -511


Grade I + II
Grade III
Count
15
12
9
6
3
0
TC- (N=32)
TC+ (N=14)
IL-1b -511+3962

B


Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
-511T- (N=34)
-511T+ (N=25)
0
50
100
150
200
250
Time to distant recurrence (months)
-511+3962TC- (N=34)
-511+3962TC+ (N=17)
Time to distant recurrence (months)
-511T- (N=34)
-511T+ (N=25)
0
100
200
300
400
Time to B CA Death (months)
-511+3962TC- (N=34)
-511+3962TC+ (N=17)
Time to B CA Death (months)

prognosis and survival were not attributable to variability in relative intratumoral IL-1β mRNA levels (Table 6.2).

The association of the IL-1β -511+3962TC haplotype with decreased tumor grade supports the finding of Smith et al. (2004) who observed a trend for decreased tumor grade in IL-1β -511T/C individuals as compared to C/C homozygotes, from a study of 144 European breast carcinoma patients [210]. This study did not examine other SNPs within the IL-1β gene sequence and it is likely that the influence of IL-1 gene polymorphism on disease progression will be dependent on the combination of alleles carried by the patient. Thus, it is unclear whether the association of IL-1β with prognosis and survival is via alterations in IL-1β production or linkage with an unidentified gene.

We did not observe an association of IL-1β SNPs with IL-1β mRNA levels (Table 6.2), however, the IL-1β -511T associates with enhanced LPS-induced IL-1β secretion of *ex* vivo stimulated whole blood cells [295] and the IL-1β exon 5 T allele associates with increased IL-1β secretion from LPS-stimulated peripheral blood monocytes [296]. The IL-1β -511 SNP is likely to play a minimal role in the control of IL-1β production and any associations observed are likely attributable to the tight linkage of the -511 SNP with an upstream -31 polymorphism which influences the TATA box [297]. Others have demonstrated the IL-1β -511 allele has minimal effects on IL-1β secretion. Instead, levels of constitutive and LPS-stimulated IL-1β are dependent on the IL-1R antagonist, which is influenced by allelic variation in the IL-1RN gene and modulates IL-1β via interception of the IL-1 auto amplification loop through IL-1R [298]. We did not observe associations of either IL-1RN or IL-1R SNPs with relative

Table 6.2: Associations of cytokine SNPs with relative intratumoral cytokine mRNA levels in invasive breast carcinoma

Cytokine SNP	N	Relative cytokine mRNA levels		*P*
		Mean±SEM	Median	
IL-1β -511T-	32	21.3±2.4	17.8	0.765
IL-1β -511T+	23	18.6±1.8	18.1	
IL-1β -511+3962 TC-	32	21.3±2.4	17.8	0.991
IL-1β -511+3962 TC+	15	19.3±2.6	18.9	
IL-1β -511+3962 TC/TC	6	17.9±3.7	16.3	0.759
IL-1β -511+3962 TC/x	9	20.2±3.8	19.4	
IL-1β -511+3962 x/x	32	21.3±2.4	17.8	
IL-6 -174nt565 CA-	19	32.3±5.3	27.3	0.467
IL-6 -174nt565 CA+	37	26.9±2.9	23.2	
IL-6 -174nt565 CA/CA	4	40.1±5.2	37.5	0.211
IL-6 -174nt565 CA/x	33	25.3±3.1	22.3	
IL-6 -174nt565 x/x	19	32.3±5.3	27.3	
IL-10 -1082-819-592 GCC-	17	21.6±3.2	22.7	0.918
IL-10 -1082-819-592 GCC+	37	22.0±2.0	21.1	
IL-10 -1082-819-592 GCC/GCC	8	23.9±4.4	21.9	0.918
IL-10 -1082-819-592 GCC/x	29	21.5±2.2	21.1	
IL-10 -1082-819-592 x/x	17	21.6±3.2	22.7	
TGF-β Codon10Codon25 TG-	5	76.1±16.0	65.4	0.052
TGF-β Codon10Codon25 TG+	47	52.2±3.4	52.0	
TGF-β Codon10Codon25 TG/TG	19	54.4±7.3	50.5	0.152

Cytokine SNP	N	Relative cytokine mRNA levels		*P*
		Mean±SEM	Median	
TGF-β Codon10Codon25 TG/x	28	50.7±2.8	52.2	
TGF-β Codon10Codon25 x/x	5	76.1±16.0	65.4	
IL-2 -330+166 TG-	25	8.6±2.5	4.5	**0.006**
IL-2 -330+166 TG+	35	14.7±1.9	12.6	
IL-2 -330+166 TG/TG	12	15.8±3.1	14.1	**0.020**
IL-2 -330+166 TG/x	23	14.2±2.4	11.1	
IL-2 -330+166 x/x	25	8.6±2.5	4.5	
IL-2 -330+166 TT-	28	14.0±2.2	10.8	0.106
IL-2 -330+166 TT+	32	10.5±2.2	5.4	
IL-2 -330+166 TT/TT	5	6.2±3.0	4.1	0.206
IL-2 -330+166 TT/x	27	11.4±2.5	6.3	
IL-2 -330+166 x/x	28	14.0±2.2	10.8	
IFN-γ +874 T-	16	4.6±1.5	2.2	**0.007**
IFN-γ +874 T+	43	13.9±2.4	6.9	
IFN-γ +874 A/A	16	4.6±1.5	6.1	**0.010**
IFN-γ +874 A/T	31	16.9±3.1	17.2	
IFN-γ +874 T/T	12	6.4±1.3	4.6	

IL-1β mRNA levels (Table 6.2). Furthermore, as the +3962 SNP is located in exon 5 of the coding sequence, it may influence protein structure. Thus, it is possible that such polymorphisms may still play a role in influencing levels of IL-1β and detection of mRNA levels may not be truly representative of protein levels.

6.4.2 IL-6

IL-6 represents another pleiotropic pro-inflammatory cytokine that can stimulate breast tumor cell growth [299] and enhance IL-1 induced breast cancer cell motility [300]. The prognostic significance of IL-6 in breast carcinoma is variable depending on the stage of disease. Expression of IL-6 by breast tumor cells associates with decreased tumor grade and estrogen receptor expression [301] and improved survival in early stage breast carcinoma [302], while increased serum levels of IL-6 correlate with angiogenic factors and decreased patient survival in metastatic breast carcinoma patients [303, 304]. In this study, we assessed two polymorphisms located in the 5' untranslated region of the IL-6 gene; the -174 G->C transition and the nt565 G->A transition.

Comparison of IL-6 SNP allelic variants with prognostic parameters revealed the IL-6 -174nt565 CA haplotype associated with increased ER levels (Figure 6.2). Likewise, fewer IL-6 -174nt565 CA+ individuals had ER- tumors as assigned by categorical designation (Pearson's χ^2=8.2, P=0.004, data not shown). The IL-6 -174C and IL-6 nt565A alleles showed the same associations with ER as the -174nt565 CA haplotype (data not shown), as there was complete concordance between the two alleles (χ^2=60.0, P<0.0001). There were too few CA/CA homozygous tumors with ER information (N=3) to warrant genotype comparison. IL-6 SNPs did not associate with patient survival (data not shown) or intratumoral IL-6 mRNA levels (Appendix III and Table 6.2).

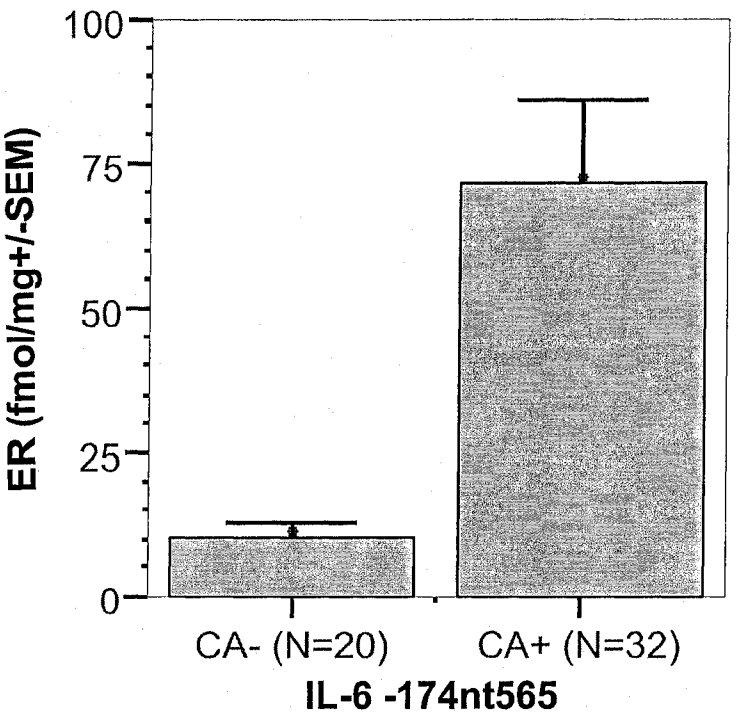


Figure 6.2: The IL-6 -174nt565 CA haplotype associates with increased ER levels in invasive breast tumors (Mann Whitney U test, *P*=0.003).

Our finding of increased ER levels in tumors carrying the IL-6 -174nt565 CA haplotype is in contrast to that of Iacopetta et al. (2004), who reported individuals of European Caucasian descent that were homozygous for the -174C allele had significantly decreased overall survival and had tumors that were more frequently of high histological grade and displayed a trend for low ER content [218]. However, the cut-off value for ER was not specificed in this study and they did not examine ER as a continuous variable. Two other studies of European Caucasians [214] and mixed ethnicity Americans [216] failed to demonstrate an association of the IL-6 SNP with ER, or other prognostic indicators. In contrast to Iacopetta et al. (2004), a study of LN positive breast carcinoma patients found IL-6 -174G/G homozygotes had significantly decreased disease-free and overall survival, that was more pronounced in the ER negative group of patients [219]. While not addressed in their study, individuals that carry the -174C allele were more frequently ER+ (32/51 (63%)) than -174G/G homozygotes (15/29 (52%)), using a cut-off value of 10% positive cells although this difference was not statistically significant (Pearson's χ^2=0.9, P=0.335).

Associations of IL-6 promoter polymorphisms with ER levels are intriguing as estradiol downregulates IL-6 production via interactions of ER with NF-IL6 and NF-κB that prevent their binding to the IL-6 promoter [305]. This modulation is likely influenced by allelic variation in the IL-6 promoter as 17-β-estradiol mediates differential effects on the activity of IL-6 -174 allelic variants [306]. Using reporter gene assays in an ER+ cell line the -174C allele had higher phorbol-myristate acetate (PMA)-induced, but not constitutive, transcriptional activity in the absence of 17-β-estradiol than the -174G allele. Addition of 17-β-estradiol corrected the inability of PMA to stimulate IL-6 -174G

allele transcription. Thus, associations of IL-6 SNPs with intratumoral IL-6 production will likely be dependent on estradiol levels, which were not assessed in this study.

These aforementioned studies assessed only the IL-6 -174 SNP, which was previously identified to influence *in vitro* and *in vivo* IL-6 levels. Fishman et al. (2000) demonstrated, using reporter gene transfected HeLa cells, that the IL-6 -174 C promoter had lower constitutive, IL-1 and LPS induced activity than the IL-6 -174G promoter. Furthermore, comparison of plasma IL-6 levels revealed IL-6 -174G/G homozygotes (n=35) had significantly increased plasma IL-6 as compared to C/C homozygotes (n=22). However Terry et al (2000) demonstrated, using transient transfection assays of IL-6 haplotype constructs, that the genetic control of constitutive and IL-1-induced IL-6 production was controlled by the combined effects of the -174G>C, -373A_nT_n, -572G>C and -597G>A (equivalent to nucleotide 565 of the gene sequence) polymorphisms and identified cell-type specific differences in this control [202]. Thus, in the context of breast carcinoma, the control of intratumoral IL-6 levels is complex and likely influenced by the cell types present in the immune infiltrate as well as tumor cell ER expression and estradiol levels.

6.4.3 IL-10

IL-10 represents an important immunomodulatory cytokine and is present at high levels in breast carcinoma [307]. Although IL-10 can suppress anti-tumor immune responses via suppression of the pro-inflammatory effects of antigen presenting cells and inhibition of T cell activation, experimentally IL-10 can also inhibit tumor growth via NK cell activation, blockade of angiogenesis and induction of metalloproteinase inhibitors

[308]. We evaluated three SNPs of the IL-10 promoter; the -1082 G->A transition, the -819 C->T transition and the -592 C->A transition.

Comparison with prognostic parameters revealed the IL-10 -1082-819-592 GCC haplotype associated with decreased levels of ER and PR (Figure 6.3A). Likewise, by categorical assignment, individuals that carry the IL-10 -1082-819-592 GCC haplotype were more frequently ER- (χ^2=6.0, *P*=0.015, data not shown). ER levels were decreased in both GCC homozygotes and GCC heterozygotes (Figure 6.3B). The IL-10 -1082-819-592 GCC haplotype also associates with infiltrating ductal tumors (Figure 6.3C), which contain lower levels of hormone receptors [Mann Whitney U test, P=0.012 (ER), P=0.040 (PR); data not shown]. IL-10 SNPs did not associate with patient survival (data not shown), or with intratumoral levels of IL-10 mRNA (Appendix III and Table 6.2). This finding was not unexpected as intratumoral IL-10 mRNA levels did not associate with hormone receptor expression or tumor type in the larger group of breast carcinoma patients from which this subset were selected (Chapter 5).

Few studies have examined IL-10 SNP associations with prognosis in breast carcinoma. Although the IL-10 -592A allele associated with increased risk of breast cancer development in a study of 500 Austrian Caucasian breast carcinoma patients and 500 age matched females, it did not associate with tumor size, LN status, tumor grade, ER, PR or diagnosis age [215]. Another study of mixed ethnicity American women assessed the IL-10 -1082, -819 and -592 SNPs and did not find an association of either SNP with risk of disease or prognosis [216]. The lack of consistent associations of IL-10 SNPs with disease susceptibility and prognosis is hypothesized to be attributable to the dichotomy of IL-10 in the immune response to carcinoma, functioning as both an anti-

Figure 6.3: Associations of IL-10 SNPs with prognostic parameters in invasive breast carcinoma. **A)** The IL-10 -1082-819-592 GCC haplotype associates with decreased ER (Mann Whitney U test, P=0.004) and PR levels in invasive breast tumors (Mann Whitney U test, P=0.041). **B)** Both IL-10 -1082-819-592 GCC homozygotes and heterozygotes have decreased ER (Kruskal Wallis H test χ^2=8.7, P=0.013, Pc=0.038). While PR levels do not significantly associate with IL-10 genotype (P=0.118, Pc=0.314). Genotype groups were compared by Mann-Whitney U test, significant differences are indicated by asterisks. *P<0.05, **P<0.01. **C)** IL-10 -1082-819-592 GCC tumors are more frequently of the infiltrating ductal carcinoma (IDC) subtype (Fisher's exact 2-sided test, P=0.019). All GCC/GCC homozygous tumors are IDC (Kruskal Wallis H test χ^2=7.1, P=0.029, Pc=0.085).

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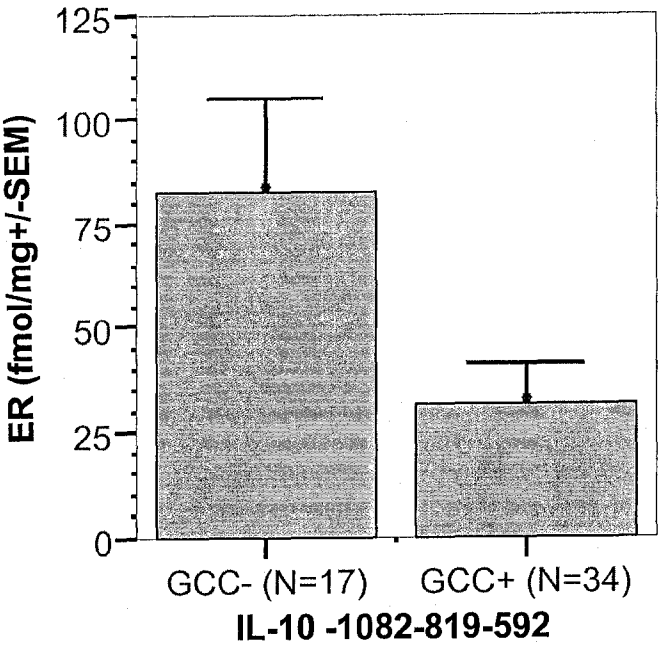
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ER (fmol/mg+/-SEM)
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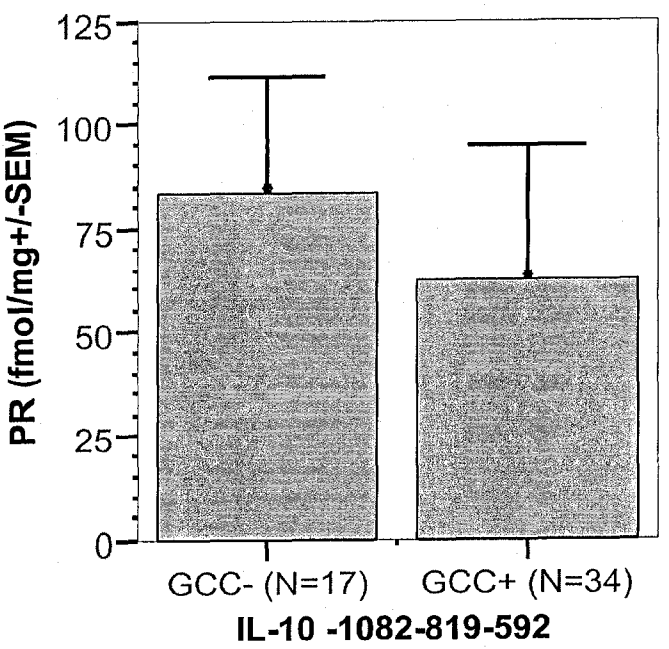
IL-10 -1082-819-592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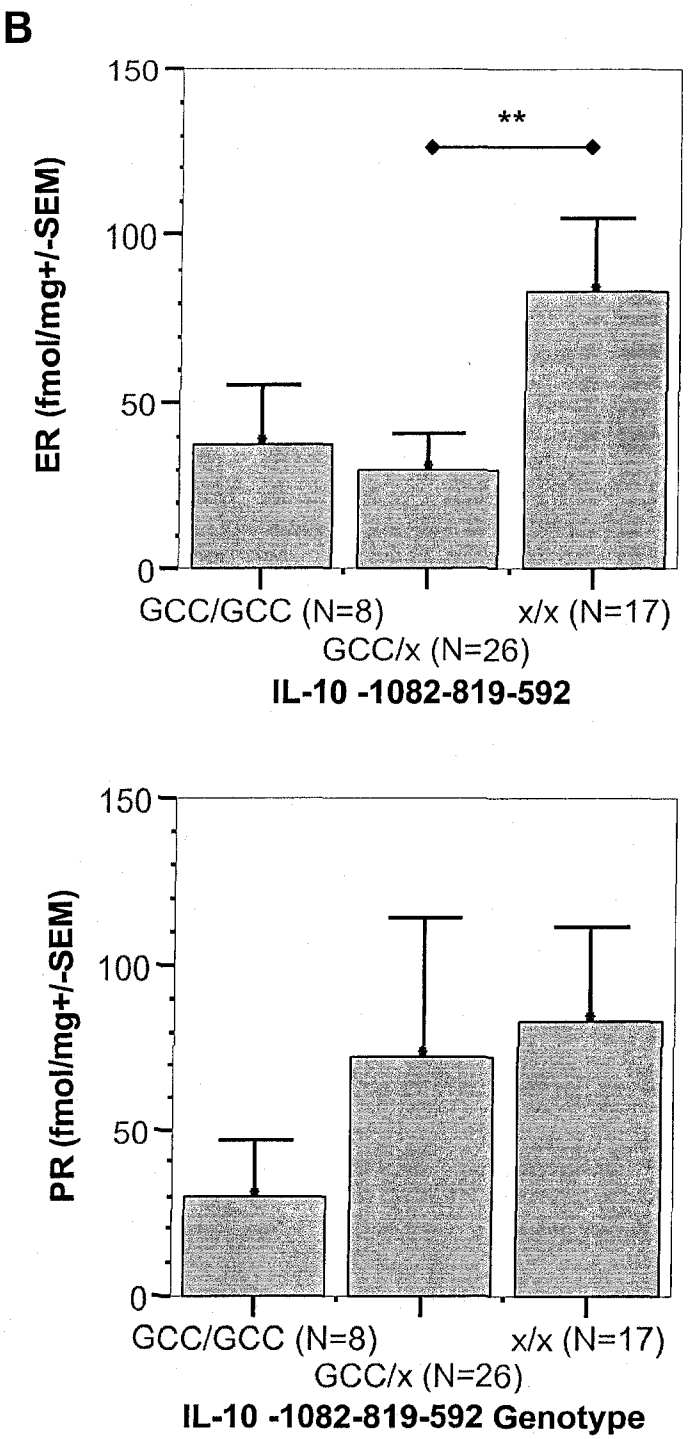

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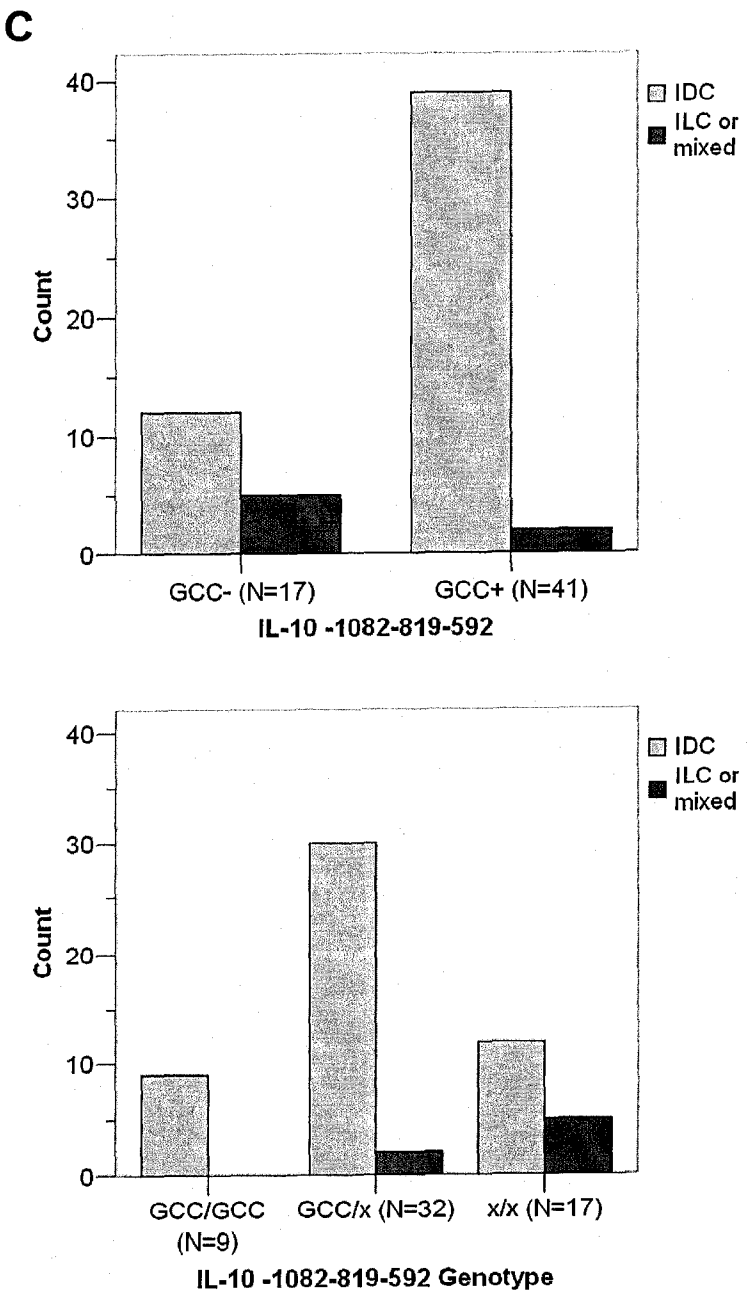

Figure 6.3 continued.

inflammatory and angiogenesis promoting cytokine [309].

The IL-10 -1082-819-592 GCC haplotype has been demonstrated to associate with increased IL-10 transcriptional activity by reporter gene transfection assay [310] and significantly higher IL-10 secretion following *in vitro* stimulation of PBMC from healthy IL-10 -1082 G/G homozygotes [311], specifically IL-10-1082-819-592 GCC/GCC individuals [206]. Furthermore, plasma levels of IL-10 are increased in healthy G/G homozygotes, however, diseased individuals do not demonstrate such an association [312]. In contrast, Warle et al. (2003) observed IL-10 -1082-819-592 GCC/GCC homozygous PBMC produced significantly lower IL-10 production following *in vitro* stimulation in both healthy individuals and liver transplant patients, using a similar mitogen stimulation protocol as Turner et al. (1997) [311]. The reasons for this discrepancy are unclear; however, they may in part reflect the influence of other upstream IL-10 genetic variants. Extended promoter haplotype analysis revealed the GCC haplotype exhibits variability in its linkage to distal promoter gene variations, which were shown to influence IL-10 production by PBMC and EBV-transformed B cells [313]. This study also identified differential effects of cell stimulus (LPS, dibutyryl-cAMP and EBV) on associations of allelic variants with IL-10 production. Thus, genetic control of IL-10 production likely involves the interaction of multiple allelic variants and will be cell-type and stimulus dependent.

6.4.4 TGF-β1

TGF-β1 represents another immunomodulatory cytokine expressed at high levels in breast carcinoma that exhibits both tumor suppressing and tumor promoting effects. TGF-β1 inhibits tumor growth at the early stages of tumor development, but later,

autocrine TGF-β signaling in tumor cells can promote tumor growth, invasion and metastasis [314]. Indeed, breast carcinoma patients with advanced disease have elevated plasma TGF-β1 [315] and intratumoral TGF-β1 protein expression [316] associates with decreased survival in breast carcinoma patients. We evaluated two SNPs of the TGF-β1 signal sequence. The Codon 10 T->C transition results in a leucine/proline substitution at amino acid 10, while the Codon 25 G->C transition results in an arginine/proline substitution at amino acid 25.

In the breast carcinoma patients assessed in this study, TGF-β1 SNPs did not associate with prognostic parameters or survival (data not shown). However, tumors that carry the TGF-β1 Codon 10 Codon 25 TG haplotype display a trend for decreased intratumoral TGF-β1 mRNA (Table 6.2). This finding supports previous studies that found the TGF-β1 Codon 10 T allelic variant associates with decreased TGF-β1 production. HeLa cells transfected with a TGF-β1 cDNA CMV construct containing the T allele had a 2.8-fold decrease in secretion of TGF-β1 than those transfected with the TGF-β1 Codon 10 C allele CMV construct [201]. Furthermore, serum levels of TGF-β1 are lower for Codon 10 T/T homozygotes as compared to C/C homozygotes [317].

Other groups have also observed a lack of association of TGF-β1 Codon 10 or Codon 25 SNPs with prognostic parameters in breast carcinoma patients [216, 217, 318], although the TGF-β1 Codon 10 C/C homozygous individuals have increased incidence of metastasis and decreased survival, as compared to individuals that carry at least one T allele at Codon 10 [216, 318, 319]. These findings would support the *in vitro* and *in vivo* associations of the TGF-β1 Codon 10 T allele with decreased TGF-β1 and the role of high levels of TGF-β1 in disease progression [314].

6.4.5 IL-2

IL-2 is a prototypical T cell cytokine and substantial experimental and clinical evidence suggests IL-2 is crucial in the regulation of anti-tumor immunity, via its stimulatory actions on TAA-specific CTLs, NK cells, B cells and macrophages [320]. IL-2 mRNA and protein are also detected at low levels in breast carcinoma cells [321] and its expression may function as an autocrine growth factor [322]. We evaluated two SNPs of the IL-2 gene; the -330 T-> G transition of the 5' untranslated region and the +166 G->T transition of the leader peptide.

We observed an association of the IL-2 -330+166TT haplotype with large tumors, when assessed as a categorical variable (Figure 6.4A), and IL-2 -330+166TT individuals displayed a trend for increased tumor size when tumor diameter was assessed as a continuous variable (Mann Whitney U test P=0.076, data not shown). This statistically significant difference was not observed when IL-2 -330+166 TT genotype categories were compared (Figure 6.4A). Other IL-2 SNP alleles/haplotypes did not associate with prognostic parameters (data not shown). Furthermore, there were no observed differences in patient survival with respect to IL-2 SNPs (data not shown). Despite the observed association with tumor size, the IL-2 -330+166TT haplotype did not associate with intratumoral IL-2 mRNA levels. This was somewhat unexpected as IL-2 mRNA levels negatively correlated with tumor size in the larger group of breast carcinoma patients from which this subset were selected (Chapter 5). However, patients that carried the IL-2 -300+166 TG haplotype had significantly higher IL-2 levels (Table 6.2), reflected by both

Figure 6.4: Associations of IL-2 SNPs with prognostic parameters in invasive breast carcinoma. **A)** The IL-2 -330+166 TT haplotype associates with large tumors (χ^2=4.4, *P*=0.036) and a trend of association was observed with -330+166 TT genotype groups (χ^2=4.6, *P*=0.101, *Pc*=0.273). **B)** Median and Mean ± SEM of IL-2 mRNA levels in tumors stratified by relative numbers of infiltrating CD3+ TIL. The IL-2 -330+166 TG haplotype associates with increased intratumoral IL-2 mRNA in tumors with no CD3+ TIL (Mann-Whitney U test, *P*=0.024), small to moderate numbers of CD3+ TIL (Mann-Whitney U test, *P*=0.030), but not in tumors with large numbers of infiltrating CD3+ TIL (Mann-Whitney U test, *P*=0.755), as detected by indirect immunohistochemistry.

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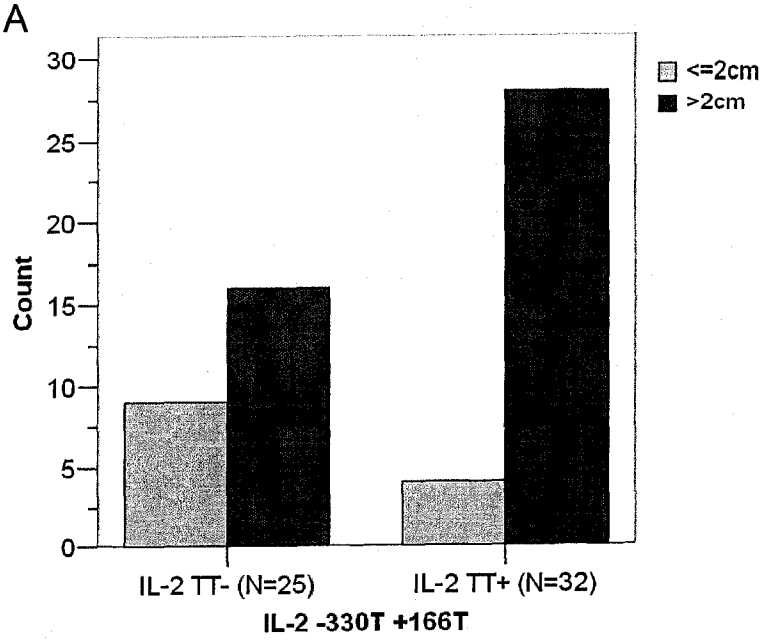
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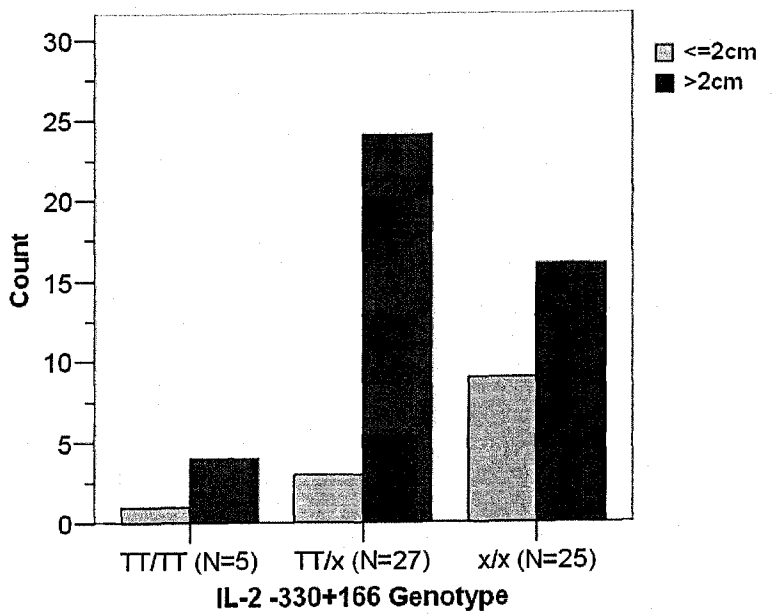
IL-2 -330T +166T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IL-2 -330+166 Genotype

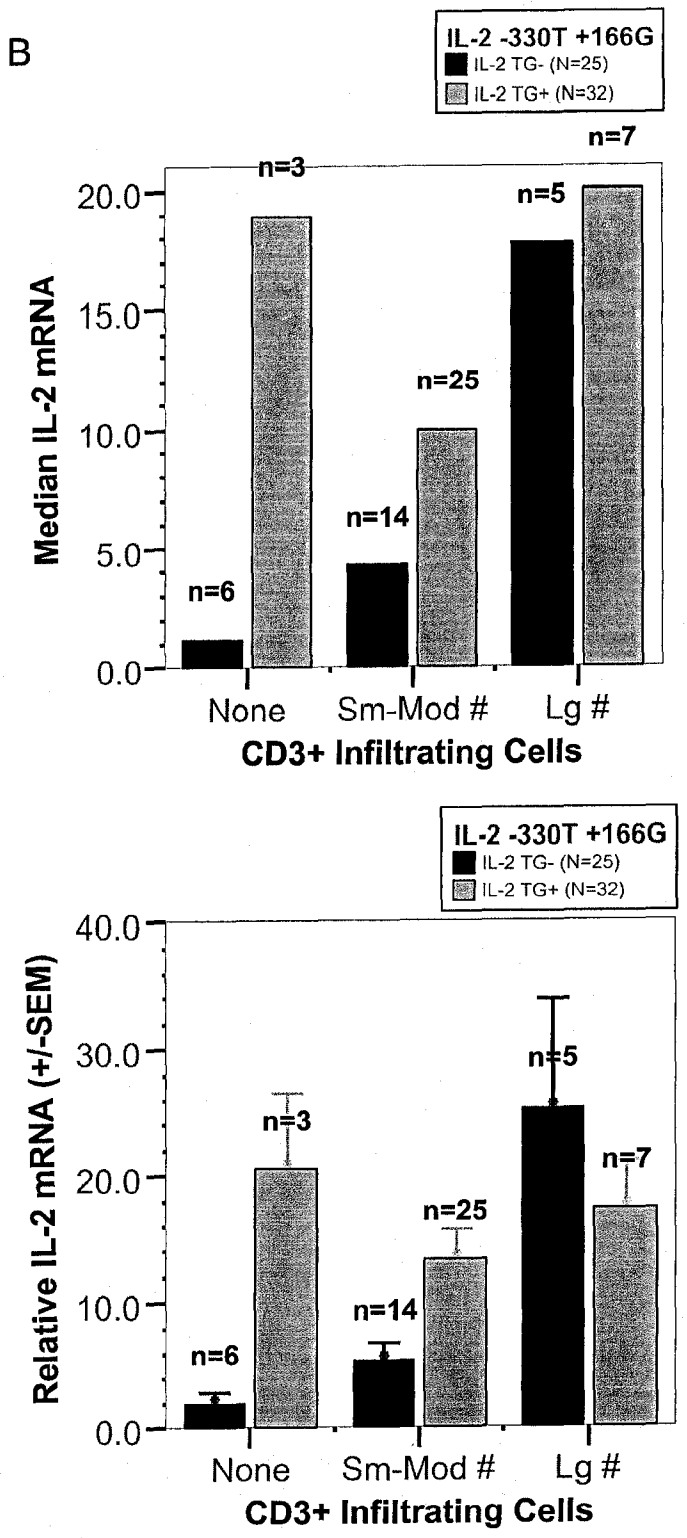


Figure 6.4 continued.

TG/TG homozygotes and TG/x heterozygotes and 9/13 (69%) small tumors carried the IL-2 -330+166 TG haplotype as compared to 23/44 (52%) large tumors, although not statistically significant (χ^2=1.2, *P*=0.279).

There have been no published reports examining IL-2 SNPs and disease susceptibility or prognosis in breast carcinoma. However, the effect of the IL-2 -330 SNP on cytokine production has been investigated. Hoffmann et al. (2003) observed PBMC from healthy individuals homozygous for the IL-2 -330 G allele produce increased IL-2 protein following *ex* vivo stimulation via TCR engagement [206]. Using reporter gene constructs, Matesanz et al. (2004) [323] found that the Jurkat T cell line, transfected with the IL-2 -330G containing construct, produced twofold higher levels of gene expression than the -330T construct transfected cells, following PMA activation. However, they observed opposite contradictory observations when examining IL-2 mRNA as IL-2 levels were increased in IL-2 -330 T/T and T/G stimulated peripheral blood lymphocytes, from healthy controls and multiple sclerosis patients, suggesting additional factors influence the *in vivo* situation. Indeed, we observed increased intratumoral IL-2 mRNA levels in individuals carrying the -330+166 TG haplotype but not the -300+166 TT haplotype, suggesting an influencing effect of allelic variation at position +166, or an unidentified linked factor.

As we have previously demonstrated high relative intratumoral levels of IL-2 mRNA associate with increased numbers of infiltrating CD3+ TIL (Chapter 5), we further examined the influence of IL-2 SNPs within tumors subgrouped by CD3+ TIL. Stratification of primary tumors by relative numbers of CD3+ TIL revealed relative IL-2 mRNA levels were increased in individuals carrying the IL-2 -330+166TG haplotype

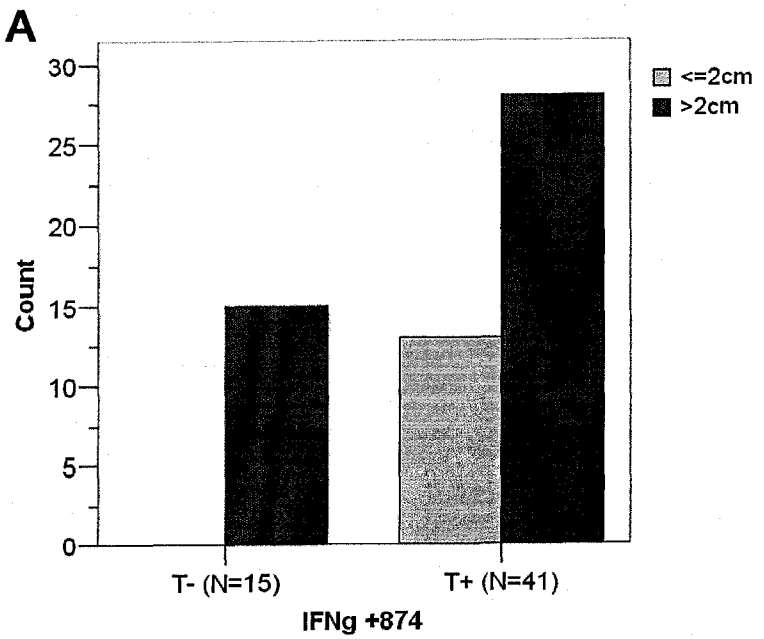
with no or small to moderate numbers of CD3+ TIL. However, in the subgroup of patients with large numbers of CD3+ TIL, relative intratumoral IL-2 mRNA levels were independent of SNPs as both TG- and TG+ individuals had elevated IL-2 mRNA (Figure 6.4B). This suggests the genetic control of IL-2 production in breast carcinoma is minimal in the presence of large numbers of infiltrating TIL. In the absence of substantial TIL, IL-2 production is attributable to other cell types; including breast tumor cells [321], which may exhibit cell type specific variability in regulatory elements that influence the effects of genetic variation on IL-2 production.

6.4.6 IFN-γ

IFN-γ is produced mainly by T_H1 cells, CTLs and NK cells and is a potent anti-cancer agent via enhancement of the anti-tumor activity of host's immune cells. Elevated intratumoral IFN-γ associates with decreased tumor size and improved patient survival in breast (Chapter 4) [256] and ovarian carcinoma [270]. Experimentally, IFN-γ is critical in mediating the anti-tumor immune response to metastatic breast carcinoma [324]. We have investigated the IFN-γ +874 T->A transistion, which correlates with a CA-repeat in intron 1 of the IFN-γ gene [325].

In our subset of breast carcinoma patients, the IFN-γ +874T allele associated with decreased tumor diameter and this was observed with both +874T/T homozygotes and A/T heterozygotes (Figure 6.5A). The IFN-γ +874T allele also associated with histological grade III tumors (Figure 6.5B). Patients that carry the IFN-γ +874T allele associated with increased intratumoral IFN-γ (Table 6.2), but this was largely attributable to A/T heterozygotes (Table 6.2). These associations support our finding that increased

Figure 6.5: Associations of IFN-γ SNPs with prognostic parameters in invasive breast carcinoma. **A)** The IFN-γ +874T allele associates with decreased tumor size (Fisher's exact 2-sided test, P=0.012), and is most pronounced in A/T heterozygotes (χ^2=8.3, P=0.015, Pc=0.044). **B)** The IFN-γ + 874T allele associates with increased tumor grade (χ^2=5.4, P=0.020), observed in both T/T homozygotes and A/T heterozygotes (χ^2=5.7, P=0.058, Pc=0.164). **C)** Median and Mean ± SEM of IFN-γ mRNA levels in tumors stratified by relative numbers of infiltrating CD3+ TIL. The IFN-γ +874T allele associates with increased intratumoral IFN-γ mRNA in tumors with small to moderate numbers of CD3+ TIL (Mann-Whitney U test, P=0.040), but not in tumors with no CD3+ TIL (Mann-Whitney U test, P=0.886), which have low levels of IFN-γ or in tumors with large numbers of infiltrating CD3+ TIL as all but one tumor with large numbers of CD3+ TIL carry the IFN- γ +874T allele (Mann-Whitney U test, P=0.500).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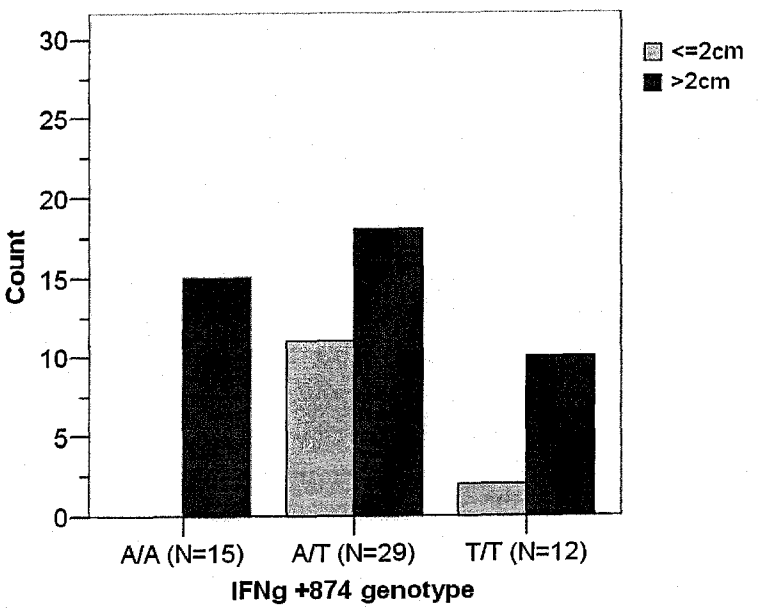
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A/T (N=29)
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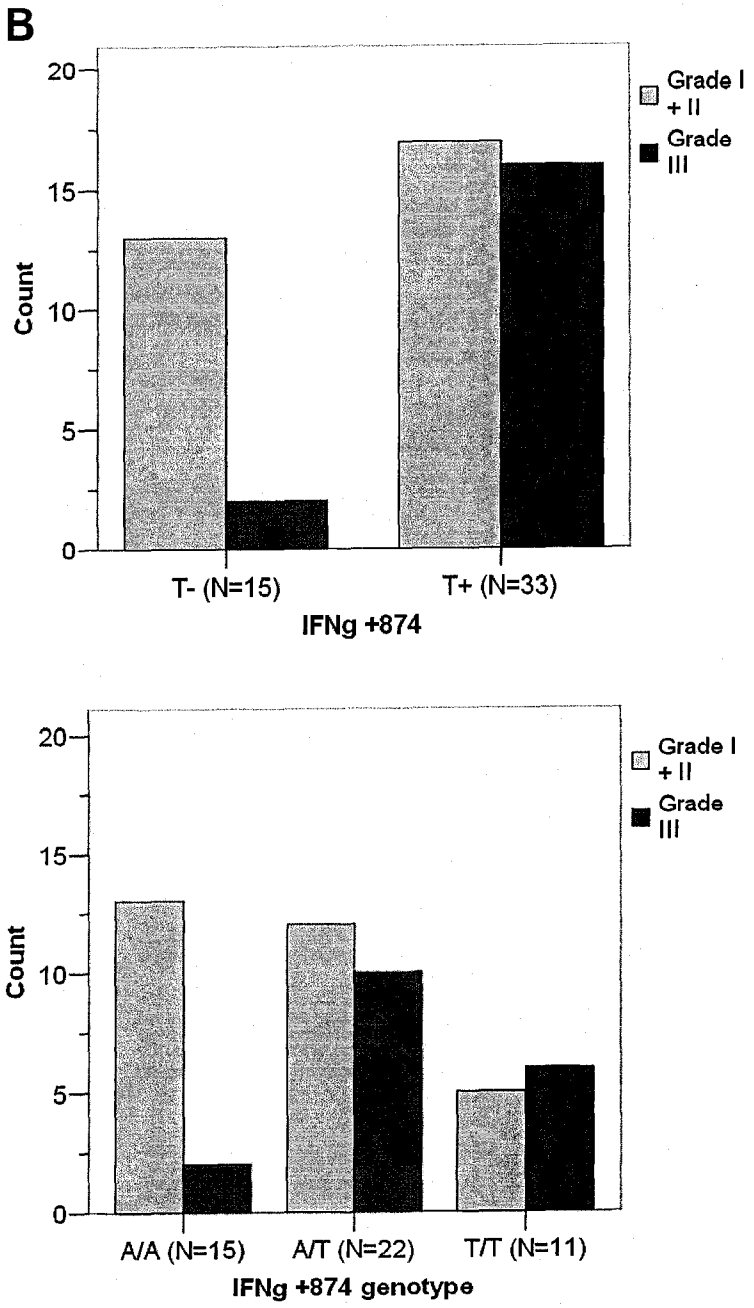
IFNg +874 genotype



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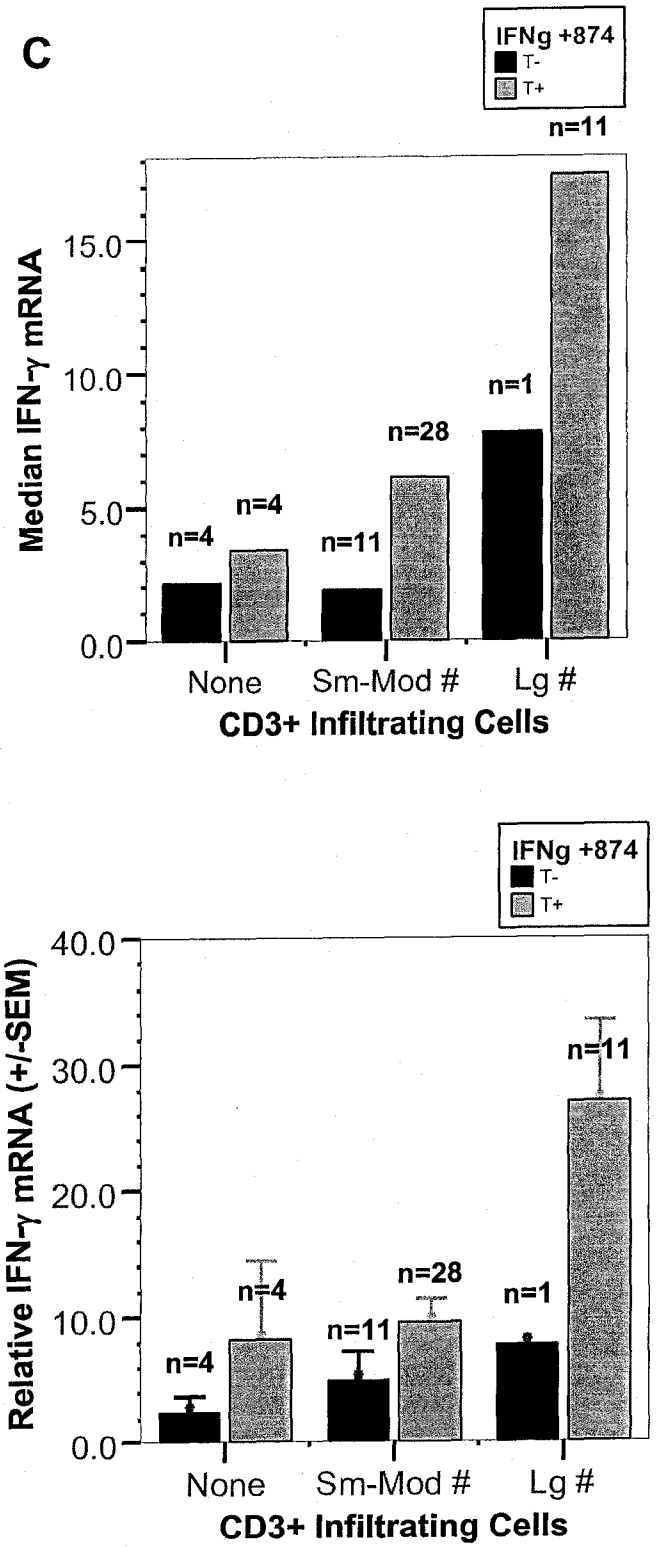

Figure 6.5 continued.

intratumoral IFN-γ mRNA levels associate with small tumor diameter and increased tumor grade (Chapter 5).

The IFN-γ +874 T/T genotype associated with increased breast cancer risk, in two small scale studies of Iranian [326] and Indian women [327], but did not predict increased risk of disease in a small study of American women of mixed ethnicity [216]. Previous studies did not demonstrate an association of the IFN-γ +874 SNP with prognostic parameters [216, 326]. However, in contrast to our study, neither tumor size nor tumor grade were examined in these patients.

The association of the IFN-γ +874T allele with increased intratumoral IFN-γ supports that of Pravica et al. (1999) who demonstrated in healthy individuals that IFN-γ +874T/T homozygotes produce more IFN-γ, following *in vitro* stimulation of PBMC with mitogen [205]. Hoffmann et al. also demonstrated increased IFN-γ secretion from PBMC of healthy individuals in both T/T homozygotes and A/T heterozygotes following mitogen stimulation and significantly higher IFN-γ secretion from T/T homozygotes compared to A/T or A/A individuals following anti-CD3/CD28 stimulation of PBMC [206]. However, contradictory reports exist as Warle et al. (2003) did not demonstrate an association of the IFN-γ +874 SNP with *in vitro* production of IFN-γ in liver transplant recipients [223] and reporter gene assays demonstrated higher activity of the IFN-γ -874A promoter as compared to the -874 T variant HepG2 hepatoma and unstimulated and PMA treated Jurkat T cells [327]. Saha et al. (2005) suggest discrepancies in reports of transcriptional activity and protein levels in relation to IFN-γ +874 SNPs may reflect the association with an unidentified variation apart from the CA repeat, which is in complete

linkage with position +874 [325] and shown to assocate with *in vitro* IFN-γ production [205] or alternatively may reflect posttranscriptional differences attributable to the CA repeat length of the IFN-γ intron region [327]. These studies suggest genetic control of IFN-γ production is far more complex than the single polymorphism at position +874 and factors produced in diseased individuals may modulate IFN-γ production in variable ways to that seen in healthy individuals.

As relative intratumoral IFN-γ mRNA also associates with numbers of CD3+ TIL (Chapter 5), IFN-γ +874 SNP associations with IFN-γ mRNA levels were assessed in tumors stratified by relative CD3+ TIL numbers. Relative intratumoral IFN-γ was slightly increased in individuals carrying the IFN-γ +874T allele with small or moderate numbers of CD3+ TIL. However, levels did not associate with the IFN-γ +874T allele in tumors with no CD3+ TIL, reflecting the decreased IFN-γ in these tumors, nor with tumors containing large numbers of CD3+ TIL, as all but one carried the IFN-γ +874T allele (Figure 6.5C). This suggests infiltrating TIL play the predominant role in determining relative intratumoral IFN-γ levels.

6.5 CONCLUSION

The small number of breast tumors examined and the semi quantitative determination of intratumoral cytokine mRNA levels limit interpretation of our findings. However, the results of this small study suggest reports of cytokine SNP associations with breast cancer susceptibility or prognosis must be interpreted with caution in the absence of information on the cytokine levels. Indeed, individuals in a diseased state do not always share the associations of cytokine gene polymorphisms with *in vitro* cytokine

production following cell stimulation or plasma levels, observed in healthy individuals [223, 312]. Few studies have evaluated the influence of genetic polymorphisms on *in vivo* cytokine production. However, establishing a link with cytokine production in the context of carcinoma and other diseased states will likely prove difficult as carcinomas are marked by inflammatory infiltrates and the interplay of other cytokines and growth factors likely override this genetic control.

Although the number of samples was too few to warrant a meaningful statistical analysis, stratification of tumors based on relative numbers of CD3+ TIL and IFN-γ or IL-2 SNPs revealed that although intratumoral cytokine mRNA levels may in part be influenced by promoter polymorphisms they are largely dependent on the relative number of infiltrating inflammatory cells. Furthermore, associations of some cytokine SNPs with prognostic parameters occurred in the absence of intratumoral cytokine variation. Thus, until the complexity of genetic control of cytokine production is elucidated rather than interpreting results based on published results of *in vitro* cytokine production studies on SNP associations with disease susceptibility and/or prognosis should be interpreted with respect to gene loci. Such associations may be independent of variation in immune responsiveness and attributable to linkage with additional cytokine gene allelic variants or an unidentified tumor suppressing or tumor-promoting gene.

CHAPTER 7: SUMMARY AND FUTURE DIRECTIONS

The studies described in this thesis have made many novel and important findings which increase the understanding of the *in situ* immune response to invasive breast carcinoma. Early studies of anti-tumor immunity focused largely on TAA-specfic CD8+ CTL and tumor cell HLA class I antigen expression. However, substantial evidence supports a role for tumor cell HLA class II expression in the anti-tumor immune response (see Section 1.7.2). To further understand this role in the context of invasive breast carcinoma, we evaluated components of the anti-tumor immune response within the tumor microenvironment. The major findings of this research project, limitations of experimental design and possibilities for future investigative study are discussed in the following sections.

7.1 Summary of major findings

In a small comparison study of an equal number of HLA-DRB1*04+ and non-HLA-DRB1*04 patients, we observed the DRβ1*04 allotype was expressed more frequently than other HLA-DRβ allotypes and the majority of HLA-DR+ tumors lack expression of one or more HLA-DRβ allotypes (Chapter 2). This study is the first to document differential HLA-DRβ allotype expression *in situ* in carcinoma lesions. Several factors have impeded the investigation of HLA-DRβ allospecificities in carcinoma tissue, most notably, the lack of HLA class II allotype specific antibodies at the time of earlier studies of HLA class II expression in breast carcinoma. IHC represents an effective and necessary means for evaluating HLA antigen expression in tumor tissues as studies have

shown mutations in genes that encode HLA class I antigens that inhibit their translation but not their transcription [229]. Similar mutations are likely to occur in HLA class II genes as studies conducted in our laboratory revealed the lack of protein expression of particular HLA class II specificities despite the presence of mRNA using a panel of breast carcinoma cell lines and cultured fibroblast-like synoviocytes [140, 267].

The discovery of differential HLA-DRβ allotype expression by breast tumor cells (Chapter 2) led to a larger study of unselected invasive breast carcinoma patients, with prognostic and outcome information, which allowed the investigation of the biological and prognostic implications of HLA-DRβ allotype expression by breast tumor cells (Chapter 3). Again, HLA-DRβ1*04 was expressed more frequently by breast tumor cells. Furthermore, HLA-DRβ1*04 expression by tumor cells associated with elevated IFN-γ mRNA levels, decreased tumor size and improved patient survival, while HLA-DRβ1*13 patients with DR-expressing tumor cells had decreased IFN-γ, elevated IL-1β, TGF-β1 and FOX-P3, LN metastasis and decreased survival. While these findings indirectly suggest the opposing influence of the DRB1*04 and DRB1*13 allotypes on patient outcome may be attributable to variability in immune responsiveness, this assumption is made based on relative intratumoral mRNA levels. Intratumoral mRNA levels provide an indication of the pattern of immune reactivity in breast carcinoma lesions, however the cellular source of cytokine mRNA was not identified in this study. Furthermore, the presence of mRNA is not necessarily indicative of protein production. Indeed cytokine mRNA and protein expression are not always perfectly correlated [183]. The lack of protein expression despite mRNA may reflect the improved sensitivity of RT-PCR,

problems with protein detection or, alternatively, post-translational modifications that prevent protein expression.

As discussed in Chapter 3, it is plausible to speculate that DRB1*04+ patients display an enhanced propensity for presentation of endogenous tumor antigen derived peptides by tumor cells, to activate TAA-specific CD4+ T_H1 cells. In support of this, at the time of surgery, DRB1*04+DR+ tumors had a smaller tumor diameter. This is in contrast to a Russian study which reported DRB1*04 associated with markers of a poor prognosis [128]. However, they only assessed carriage of HLA-DRB alleles and tumor cell expression of HLA-DRB1*04 protein was not examined. Furthermore, this discrepancy may be due to varying DRB1*04 allele distribution between the two populations. The majority of DRB1*04+ patients in this study carried the DRB1*0401 allele and in the larger subset of patients from which these samples were selected DRB1*0401 was increased in small tumors. In contrast, no difference was observed for DRB1* 0404 and although DRB1*0407 occurred at a frequency <5%, 10/11 DRB1*0407+ tumors had a tumor diameter > 2cm (data not shown). Allelic differences in DRB1*04 alleles may also influence their ability present peptides from endogenous tumor antigens due to differences in their relative affinity for CLIP, which is known to block the peptide binding groove preventing presentation of endogenously derived peptides. Certainly, DRβ1*0401 and DRβ1*0404 form less stable complexes with CLIP than DRβ1*0402 [328].

The idea that CLIP expression might repress effective anti-tumor immunity is suggested by our observation that co-expression of the peptide immunoeditor HLA-DM in HLA-DR expressing tumor cells associated with a T_H1 immune profile and improved

survival (Chapter 4). Although limited tissue availability precluded the examination of CLIP by breast tumor cells in this study, as discussed in Chapter 4, *in vitro* studies using BCCL demonstrated elevated surface CLIP in HLA-DM deficient BCCL as compared to HLA-DM+ BCCL. Importantly, increased survival and enhanced T_H1-type immunity in patients with HLA-DM expressing tumors and HLA-DRβ1*04-expressing tumors was not attributable to an association between the two factors (Fisher's exact 2-sided test, P=0.491; data not shown). This further suggests presentation of TAA-derived peptides by DRB1*0401 may not require HLA-DM-mediated exchange of CLIP, for the effective activation of T_H1 cells. This reduced affinity of alleles such as DRB1*0401 for CLIP will likely enhance the presentation of antigenic peptides, including TAA derived peptides, even in HLA-DM- cells.

Elevated intratumoral levels of the T_H1-type cytokine IFN-γ (Chapter 4) and the proinflammatory cytokine TNF-α (Chapter 5) associate with improved survival in invasive breast carcinoma patients. Although the cellular sources of cytokine production were not assessed in this study, IFN-γ is secreted in high amounts by activated T_H1, T_C1, NK and NKT cells and TNF-α can be secreted from macrophages, T_H1 and T_H2 CD4+ T cells, T_C1 and T_C2 CD8+ T cells as well as NK cells [121]. The association with large numbers of CD3+ TIL suggests a substantial contribution from TIL, most reflected by CD4+ TIL. Indeed, in an experimental model of invasive breast carcinoma, CD4+ TIL were the predominant producers of TIL-derived TNF-α [284] and TAA-specific CD4+ T_H1 cells that secrete both IFN-γ and TNF-α can be readily expanded *ex vivo* from breast carcinoma patients [329]. Since ovarian cancer patient survival was reported to be dependent on the balance of effector and regulatory cell subsets [195], survival was

assessed in patients categorized based on T_H1:T_{H2}/T_{reg} mRNA profiles (Chapter 5). There was no difference in survival when patients were stratified based on the ratio of IFN-γ to IL-4, IL-10, TGF-β1 or FOX-P3. However, a marked increased in DSS was observed when ranked intratumoral TNF-α levels exceeded TGF-β1. This suggests the balance of pro-inflammatory and immunoregulatory cytokines predict patient outcome.

As intratumoral cytokine mRNA levels associated with prognosis and survival in breast carcinoma patients (Chapter 5) and published reports suggested cytokine production was in part controlled by genetic variation, a small scale exploratory study was conducted to assess the relationship of cytokine gene polymorphisms with relative intratumoral cytokine mRNA and prognosis (Chapter 6). As discussed in Chapter 6, the results presented in this study must be interpreted with caution owing to the small scale of the investigation. However, they suggest SNPs of cytokine genes play a minor role in the control of cytokine production in breast carcinoma patients. The true significance of genetic variation in cytokine genes in the context of carcinoma is at present unclear as the full contribution of extended SNP haplotypes on cytokine production in response to various stimuli and in different cell types remains to be fully characterized. More extensive *in vitro* and *in vivo* studies are required to understand the combined effects of these factors on the genetic control of cytokine production. Until such factors are elucidated it will remain difficult to assess the biologic and prognositic significance of genetic variation in cytokine genes in the context of complex disease.

Intriguingly, expression of HLA class II and co-chaperones and the presence of TIL subsets associated with decreased age at diagnosis and lack of hormone receptor expression (Chapters 3-5). As discussed in these Chapters, this suggests a role for

hormonal control of expression of HLA class II antigens in breast carcinoma cells and/or the recruitment and activation of T cell subsets and enhancement of cytokine production. Additional roles for estradiol-ER interactions with the anti-tumor immune response are suggested by the discoveries that *in vitro* culture with 17-β-estradiol modulates cytokine and chemokine production by immature human DC and enhances the ability of mature DC to stimulate allogeneic T cell responses [330]. While these factors were not addressed in this study, they are important avenues for future investigation, to aid in unraveling the interplay of hormones and cytokines in breast carcinoma.

7.2 Study limitations

Utilizing IHC to assess HLA antigen expression in tumor tissue is not without pitfalls. In particular it relies on the subjective determination of the degree of tumor cell staining. However, in our experience, reading of immunohistochemistry slides by three independent readers showed a high level of concordance with the estimated percentages of positive tumor cells never differing by more than 10%. While it is acknowledged that the morphology of acetone-fixed tissue sections is inferior to formalin-fixed paraffin embedded tissue (FF-PET), such methods are required for the investigation of HLA antigen expression as many of the determinants recognized by HLA antigen-specific mAbs are lost in FF-PET due to the denaturing conditions of the fixation protocol. It is important to note, that detection of HLA-DRβ allotype and HLA co-chaperone molecule expression by immunohistochemistry does not exclude that they are non-functional or malfunctional because of mutations and/or changes in their conformation. Furthermore, as certain mAbs may be cell-type restricted and/or alternatively display extra reactions

depending on cell type and/or assay conditions, preliminary research and development studies investigated several fixation protocols and all antibodies were titrated for optimal detection of HLA allospecificities on breast cancer cell lines, at levels comparable to homozygous B cell lines (data not shown), using the same IHC procedure employed for breast carcinoma tissue sections. To further compensate for affinity differences in HLA-DRβ allotype specific mAbs, infiltrating inflammatory cells and stromal cells served as intrinsic controls. The possibility of cell-type specific reactivity was eliminated by testing antibodies on a panel of cell types that had been molecularly typed for HLA-DRB alleles (Chapter 2). Antibodies that did not conform to these guidelines were eliminated from the study. To decrease any discrepancy in detection of expression of HLA antigens and HLA co-chaperones that might be attributable to antibody affinity differences, antibodies directed against generic HLA-DR, HLA class I, Ii and HLA-DM were titrated for optimal reactivity against acetone-fixed cytocentrifuge preparations of B cell lines and IFN-γ treated BCCL. In addition, all antibodies directed against cellular differentiation antigens were titrated using acetone-fixed PBMC cytocentrifuge preparations (data not shown).

Limited tissue availability precluded immunohistochemical cytokine determination. Thus, semi-quantitative RT-PCR was utilized to determine the cytokine mRNA profile of the breast tumor lesions. RT-PCR allows for more sensitive determination of cytokine production *in situ* than immunohistochemistry and preliminary experiments demonstrated RT-PCR was much more efficient than ribonuclease protection assays which required greater amounts of RNA and were more time consuming. To control for variation in β-actin levels in tumor cells, all PCR reactions included a control cell line and breast carcinoma samples giving a β-actin level <25%

that of control cell line were eliminated from analysis. Furthermore, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control was also run with all IL-1β, IL-6 and FOX-P3 reactions and natural log-transformed ratios were positively associated by Pearson's correlation (IL-1β: r = 0.72 , $P<0.0001$; IL-6: r = 0.90 , $P<0.0001$; FOX-P3: r = 0.95 , $P<0.0001$, data not shown).

It is important to acknowledge, the breast carcinoma samples assessed by IHC and/or RT-PCR represent only a portion of the total breast tumor, which can have variability in clonal distributon of tumor cells. This study was also limited by its retrospective nature and restricted tissue availability. As previously discussed, associations of HLA class II expression and infiltrating T cells with earlier age at diagnosis and lack of estrogen receptor expression on tumor cells imply a role for hormonal regulation. However, levels of intratumoral or circulating estradiol were not determined in the patients utilized in this study and information on patient menopausal status was not available from the MBTB. In addition to standard hormone production, intratumoral enzymes such as aromatase, 17-β-hydroxysteroid dehydrogenase and estrone sulfatase regulate the production and metabolism of estrogens [331]. The use of the charcoal-dextran ligand binding assay to determine tumor cell hormone receptor expression of different histological subtypes of breast carcinoma can be problematic as lobular carcinomas can be falsely considered hormone receptor negative by biochemical assay as there are often fewer tumor cells dispersed in the sample [332]. However, in the subset of patients described in this thesis, primary tumors of ILC type had mean levels of 63 fmol/mg for ER and 117 fmol/mg for PR as compared to 35 fmol/mg and 47 fmol/mg, respectively, for IDC tumors. In addition, using a cutoff of 10 fmol/mg, only 2/13

(15.4%) ILC tumors were ER- and 3/13 (23.1%) PR- compared to 39/81 (48.1%) ER- and 29/81 (35.8%) PR- IDC tumors.

Furthermore, information on patient ethnicity was not available from the MBTB. While the majority of patients are likely of European Caucasian descent, a proportion of patients are also likely to be of North American Indian and Asian descent. Ethnic groups display differences in their distribution of HLA alleles [333] as well as cytokine SNPs [334]. Ethnic differences can affect patient outcome and may have introduced bias in the results of this study. However, multivariate analysis was employed in this study to control for clinicopathological parameters, which may have varied by ethnicity.

7.3 Future Directions

The work described in this thesis has provided insight into the complexity of the immune response to carcinoma. Although the studies described are largely descriptive, owing to the nature of the sample material, they have allowed for the generation of several hypotheses that can be further investigated:

1) Multiple mechanisms underlie the lack of HLA-DR and/or co-chaperone expression in breast carcinoma.

As discussed in Chapters 2 and 3, the mechanisms responsible for differential HLA-DRβ allotypic expression have not yet been elucidated. They are likely to include defects in CIITA or other transcription factors, epigenetic modifications, LOH, point mutations and variability in expression of cytokine receptors. Isolation of tumor cells

from breast carcinoma lesions will allow investigation of the mechanisms responsible for lack of expression of HLA class II and/or co-chaperones in breast carcinoma lesions. Enzyme disruption of tumors, followed by flow cytometry is a potential means to isolate tumor cells for further evaluation; however, due to advances in breast cancer diagnosis the size of available fresh breast carcinoma tumors available for such studies is minimal. Therefore, optimizing techniques such as laser capture micro-dissection, for isolation of tumor cells from archival FF-PET would allow further investigation of such mechanisms in a large series of breast carcinoma lesions.

2) Differential expression of HLA-DRβ allotypes represents an immune evasion strategy

As discussed in Chapter 3, lack of expression of a particular HLA-DRβ allotype by HLA-DR+ tumor cells may be reflective of immune-mediated selective pressure and the outgrowth of immune escape variants that have down-regulated the relevant HLA-DR allele. The finding that differential HLA-DRB allotype expression is a frequent phenomenon in HLA-DR$^+$ breast carcinomas infiltrated by CD4+ T cells suggests it may be in response to immune selective pressure. In support of this, all secondary lesions show differential DRβ allotypic expression. However, the primary and secondary lesions examined in this study were not from the same patient. The recent advances in tumor bank sample and data collection make the comparison of primary and recurrent tumors from the same patient a realistic possibility. Future immunohistochemical studies aimed

at investigating expression of HLA-DRβ allotypes in primary and secondary lesions obtained from the same patient may provide insight into whether allotype loss correlates with disease progression.

Contentious to this hypothesis is the finding that HLA-DRβ1*04 expression by tumor cells associates with improved prognosis and the existence of T_H1-dominated immunity. Reasons for enhanced expression of DRβ1*0401 are unknown and are unlikely to be explainable by differences in antibody affinity (Section 7.2). As discussed in Chapter 3, increased DRB1*0401 expression may involve differential targeting of HLA-DRβ1*0401 molecules through the endocytic pathway and differential peptide loading of DRβ1*0401 molecules, via heat shock protein interactions [261]. Co-localization experiments in HLA-DRB typed BCCL that express HLA-DRβ allotypes with heat shock proteins and markers of the endocytic pathway would offer insight into whether such differences exist in breast epithelial cells. Elucidating the mechanisms responsible for elevated HLA-DRβ1*04 expression may provide further insight into the control of differential HLA-DR*β* allotype expression in carcinoma lesions.

3) 17-β-estradiol and intratumoral cytokines modulate HLA class II expression by tumor cells

The negative association of HLA-DR expression and diagnosis age and ER levels suggest a role for hormonal regulation of HLA-DR and mechanisms for such control have been discussed (Chapter 3-4). However, this hypothesis is speculative as

information on menopausal status and relative hormone levels were not available for the study subjects. Studies conducted in our laboratory, demonstrated both constitutive and IFN-γ-induced HLA-DR expression by BCCL are modulated in a cell line specific manner by steroidal and non-steroidal estrogens [335]. As cell line specific differences were observed that extended beyond ER status, it suggested additional factors are responsible for the hormone mediated control of HLA-DR expression by tumor cells. Future studies employing the same cell line with or without transfected ER will provide further insight into the specific control of HLA-DR expression via 17-β-estradiol-ER complexes. As Ii and HLA-DM also associated with decreased diagnosis age and ER levels were lowest in tumors that co-express HLA-DR and Ii, elucidating the effects of 17-β-estradiol on HLA co-chaperone expression are also warranted. Furthermore, the association of HLA-DR expression with diagnosis age was somewhat variable based on the HLA-DRβ alleles carried by the patient. Thus it will be useful to investigate whether there are HLA-DRB allelic differences in the molecular control of HLA-DR expression via estradiol-ER complexes.

The association of tumor cell expression of HLA-DR and the co-chaperones Ii and HLA-DM with elevated levels of T_H1 cytokines (Chapter 4), likely reflects their upregulation in response to elevated intratumoral IFN-γ, which is a potent inducer of HLA class II antigens on BCCL [134]. Furthermore, as discussed in Chapter 3, the increased expression of HLA-DRB1*04 may in part reflect elevated IFN-γ and decreased TGF-β1, which can suppress IFN-γ induction of HLA-DR via Smad3 dependent

inhibition of CIITA promoter IV activity [260]. However, *in vitro* studies conducted in our laboratory have demonstrated that although TGF-β1 suppresses IFN-γ-induced HLA-DR expression in some BCCL, it is augmented in others, possibly via a Smad-independent signaling pathway [335]. The association of HLA-DRβ1*13 expression with elevated IL-1β in the absence of high intratumoral IFN-γ levels, suggests a role for IL-1β in the induction of HLA-DR expression in invasive breast carcinomas. As previously discussed, *in vitro* studies have demonstrated IL-1β can induce HLA-DR expression in breast carcinoma cells [135]. Whether this induction is modulated by other cytokines is not known and could be examined in future studies. This modulatory effect is likely dependent on the hormone milieu as 17-β-estradiol can inhibit IL-1 induced HLA-DR expression in a BCCL [135], although the mode of inhibition is unknown. Elucidating the mechanisms responsible for hormonal and cytokine mediated control of HLA-DR and HLA class II co-chaperones using BCCL will provide further insight into the crosstalk between steroid receptor and cytokine signaling in breast carcinoma.

Cytokine induced expression of HLA-DR and co-chaperone molecules is also likely to be dependent on the relative expression levels of cytokine receptors on breast tumor cells. As discussed in Chapter 4, induction of HLA-DM on BCCL demonstrates a dose dependency in response to IFN-γ. Furthermore, not all tumors with elevated IFN-γ expressed HLA class II antigens. In addition to the mechanisms discussed above, this lack of expression may reflect altered expression of IFN-γ receptors on breast tumor cells. Further investigation of tumor cell expression levels of IFN-γ, TGF-β1 and IL-1β

cytokine receptors in HLA class II negative breast tumors would address this as a possible mechanism.

4) 17-β-estradiol modulates TAA-specific T cell activation and cytokine production

As discussed in Chapter 4, the negative association of HLA-DR with diagnosis age and ER may be indirect and attributable to their association with infiltrating T cells which show similar negative relationships (Chapter 5). Thus, in the absence of competing ER on tumor cells, circulating estradiol may bind ER on TAA-specific effector T cells and T_{reg} cells driving their activation and cytokine secretion, as demonstrated in murine studies [264, 265]. To investigate whether 17-β-estradiol mediates similar effects in the context of breast carcinoma, it would be useful to examine the influence of 17-β-estradiol on proliferative and cytokine responses of TAA-specific T cell lines (TCL) or *ex vivo* isolated TIL or PBMC from breast carcinoma patients.

5) HLA-DRβ allotypes influence immune responsiveness

The results of Chapter 3 suggest that variability in survival of HLA-DRB1*04 and HLA-DRB1*13 patients may be explained by variation in immune responsiveness. However, as previously discussed, this speculation is made based on relative levels of whole tumor lysate cytokine mRNA. *In vitro* studies assessing the activation and cytokine production of CD4+ TIL in response to TAA-expressing tumor cells and APC expressing the relevant HLA-DRβ allotype could be employed to test this hypothesis.

Furthermore, phenotyping of the CD4+ TIL in DRB1*04 and DRB1*13 expressing tumors to determine if they are producing T_H1, T_H2 or T_H3/T_{reg} type cytokines, may provide further *in vivo* evidence to support this hypothesis.

6) Breast tumor cells that co-express HLA-DR, Ii and HLA-DM can function as efficient APC

The association of DR+Ii+DM+ tumors with large numbers of infiltrating T cells, high levels of T_H1 cytokines and improved survival warrants further investigation. It is plausible that the association is merely due to high IFN-γ levels leading to induction of HLA-DM expression, however, studies have shown HLA class II+ tumor cells can function as APC (discussed in Section 1.7.2). As discussed in Section 1.7.1, although the absence of CD80 and CD86 co-stimulatory molecule expression on tumor cells may result in T cell anergy [163], a majority of BCCL express the co-stimulatory molecule CD40 [140]. Immunohistochemical assessment of CD40 expression by tumor cells in a small sample of invasive breast tumors (N=8) revealed 2/3 HLA-DR+ tumors express CD40, as compared to 0/5 HLA-DR- tumors express CD40 (Oldford, unpublished observations). Thus, expression of CD40 or other co-stimulatory molecules expressed on breast tumor cells may provide the second signal necessary for T cell activation in the context of breast carcinoma. Furthermore, as previously discussed, initial naïve TAA-specific T cell activation is likely via DC, which present tumor antigen derived peptides in the draining lymph node and such antigen-specific T cells have less stringent requirements for subsequent re-activation at the tumor site [164]. Future studies aimed at elucidating the influence of co-chaperone expression on the antigen presenting capacity

of breast tumor cells, utilizing TAA-specific TCL are warranted. As previously discussed, HLA-DRβ allotypes exhibit varying affinities for CLIP, thus assessing the relative dependence of individual HLA-DRβ allotypes on HLA-DM for TAA-derived peptide presentation is necessary.

Furthermore, following the hypothesis that tumor cells that co-express HLA-DM and HLA-DR may function as effective antigen presenting cells, facilitating the induction of effective T_H1 anti-tumor immunity (Chapter 4), it is possible that deficient antigen presentation contributes to the poor survival of breast carcinoma patients with DR+Ii+DM- tumors. *In vitro* studies have shown MHC class II+Ii+ SAI sarcoma cells are incapable of presenting endogenously synthesized model tumor antigen targeted to the endoplasmic reticulum or plasma membrane as well as exogenously provided antigen. Co-expression of DM in these tumor cells facilitated antigen presentation of endogenously synthesized antigen targeted to the plasma membrane and exogenous antigen [336]. Thus, it is plausible similar phenomenon occur in epithelial cells whereby efficient presentation of endogenous tumor antigens by DR+Ii+DM+ breast tumor cells may be dependent on the cellular localization of the tumor antigen. Similar studies conducted using BCCL expressing varying combinations of HLA class II and co-chaperone molecules may provide further insight into the control of HLA class II mediated presentation of endogenous TAA.

7) Effective anti-tumor immunity is dependent on the balance of co-existing effector and immunoregulatory mechanisms

The use of total tumor mRNA determination to assess immune responsiveness is an attractive strategy as it is would provide a more economical and time-saving approach over immunohistochemical quantification for large scale studies of carcinoma patients. However, it is acknowledged this approach is limited by the lack of information on the cell subsets producing the relevant transcripts as well as their location within the tumor mass. At the time of initiation of these studies, monoclonal antibodies to FOX-P3 were not available. Thus, relative FOX-P3 mRNA levels were assessed as an indirect measure of infiltrating T_{reg}. Studies on a subset of ovarian carcinoma patients have reported high bulk tumor IFN-γ independently associates with improved survival [270], while high relative mRNA levels of FOX-P3 independently predict poor patient survival [276]. Although we have found high IFN-γ associates with improved survival in breast carcinoma (Chapter 4), FOX-P3 mRNA levels did not predict poor patient survival (Chapter 5). FOX-P3 mRNA levels also did not predict patient survival in a group of 98 colorectal carcinoma patients, although the criterion for cut-point determination was not specified [337]. Discrepancies may be methodological as we have used semi-quantitative end-point RT-PCR to determine relative mRNA levels, in contrast to the real-time PCR methodology of the aforementioned studies [270, 276]. However, the studies in ovarian carcinoma employed the optimal/minimal *P*-value approach to obtain their cut-points for elevated IFN-γ and FOX-P3, which can introduce bias in the survival estimates by inflating the Type I error rate [277]. In an attempt to overcome these limitations, we also assessed each mRNA as a continuous variable using univariate Cox regression analysis

(Chapter 5). Although FOX-P3 mRNA levels did not associate with patient survival, we observed that the ratio of the pro-inflammatory cytokine TNF-α and the immunoregulatory cytokine TGF-β1 was an independent predictor of survival, although the cell subsets producing these cytokines were not identified (Chapter 5).

A more extensive understanding of the complexities of effector T cell and T_{reg} cell interaction within the tumor environment is required to fully understand the balance of the two in anti-tumor immunity. Studies in ovarian carcinoma suggest the anti-tumor immune response is dependent on the relative proportions of effector CD8+ TIL and CD4+CD25+FOX-P3+ T_{reg} cells [195]. Furthermore, recent evidence suggests understanding this balance will involve expanded phenotypic characterization of infiltrating TIL subsets as the presence of large numbers of effector memory CD45RO+ TIL independently predict improved survival in colorectal carcinoma patients [338]. However, this study discussed associations in terms of CD8+CD45RO+ effector memory TIL, and failed to discuss their results in terms of CD4+ TIL subsets which also express CD45RO. Recently, studies have identified IL-17 producing T cells (T_H17) with important roles in autoimmune tissue destruction [339] yet the roles of these cells in the anti-tumor immune response remain to be elucidated. Advances in the field of HLA class II multimer analysis will facilitate their use in the identification of TAA-specific TIL, which can be isolated, using techniques such as LCM, and further characterized. Thus, extensive phenotyping and quantification of individual TIL subsets will likely determine the threshold of the balance of effector and immunoregulatory TIL subsets in determining activation or suppression of the *in situ* anti-tumor immune response.

7.4 Concluding Remarks

Much remains to be studied to expand our understanding of the breakdown in anti-tumor immunity that leads to tumor outgrowth. A full understanding of the interplay of immune effector cells and tumor cells is necessary before successful immunotherapeutic strategies can be designed and employed. The results of this study may have important implications for the design of such therapies. It is evident that successful immunotherapy will involve evoking multiple immune effectors and the maintenance of effective long term memory. As CD4+ T cells are crucial for the induction and maintenance of several anti-tumor effector mechanisms (discussed in Section 1.8.2) several avenues to exploit anti-tumor immunity currently under investigation involve evoking anti-tumor CD4+ T_H cell responses. Such methods include genetically engineered tumor cells and DC based immunotherapies [340]. However, as described in Chapter 3, there are clearly differences in immune responsiveness based on the HLA-DRB alleles carried by the patient. Furthermore, the expression of the peptide editor HLA-DM in HLA class II+ tumor cells associates with improved patient outcome possibly via enhanced presentation of TAA-derived peptides (Chapter 4). Successful immunotherapeutic regimes will surely depend on knowledge of the HLA class II alleles carried by the individual and a thorough understanding of how HLA-DRβ allotypes and HLA class II co-chaperone molecules influence CD4+ T cell subset activation. It is anticipated that future studies aimed at clearly elucidating these relationships will have important implications for design and implementation of effective prophylactic and therapeutic vaccine strategies.

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Appendix I

Associations of HLA-DRB alleles carried by breast carcinoma patients with prognostic parameters in primary tumors

AI Associations of HLA-DRB alleles carried by breast carcinoma patients with prognostic parameters in primary tumors

To demonstrate that the primary tumors selected for immunohistochemistry (IHC) evaluation were representative of the entire set of primary tumors, for which outcome information was available, associations of HLA-DRB alleles with prognostic parameters were assessed in both tumor sets (Tables AI.1-AI.16).

Table AI.1: HLA-DRB allele associations with age of diagnosis in breast carcinoma patients.

	Primary Tumor Outcome Set[†]			Primary Tumor IHC Set[‡]		
	N	Mean Dx Age (± SEM)	$P^{§}$	N	Mean Dx Age (± SEM)	$P^{§}$
Total	114	59.6 ± 1.4	-	99	58.8 ± 1.5	-
DRB1*01-	98	58.6 ± 1.5	0.066	85	57.5 ± 1.6	0.036
DRB1*01+	16	65.8 ± 3.1		14	66.2 ± 3.1	
DRB1*03-	96	58.4 ± 1.5	0.052	83	57.6 ± 1.6	0.075
DRB1*03+	18	65.7 ± 3.2		16	64.6 ± 3.5	
DRB1*04-	65	59.9 ± 1.9	0.812	59	59.3 ± 2.0	0.639
DRB1*04+	49	59.2 ± 1.9		40	57.9 ± 2.1	
DRB1*0401-	89	59.5 ± 1.6	0.858	79	58.6 ± 1.7	0.838
DRB1*0401+	25	60.0 ± 2.8		20	59.4 ± 2.9	
DRB1*07-	83	58.8 ± 1.6	0.371	72	58.0 ± 1.7	0.388
DRB1*07+	31	61.6 ± 2.5		27	60.8 ± 2.7	
DRB1*11-	96	59.6 ± 1.5	0.978	82	58.8 ± 1.6	0.973
DRB1*11+	18	59.7 ± 3.3		17	58.7 ± 3.4	
DRB1*13-	93	61.0 ± 1.5	0.031	78	60.2 ± 1.7	0.056
DRB1*13+	21	53.4 ± 2.7		21	53.4 ± 2.7	
DRB1*15-	82	59.4 ± 1.6	0.803	73	58.7 ± 1.6	0.971
DRB1*15+	32	60.1 ± 2.7		26	58.9 ± 3.1	
DRB3-	59	59.9 ± 1.9	0.829	50	58.8 ± 2.1	0.966
DRB3+	55	59.3 ± 2.0		49	58.7 ± 2.0	
DRB3*01-	89	58.5 ± 1.5	0.137	76	57.6 ± 1.7	0.159
DRB3*01+	25	63.4 ± 2.8		23	62.5 ± 2.9	
DRB3*02-	89	60.7 ± 1.5	0.118	76	59.3 ± 1.7	0.466
DRB3*02+	25	55.6 ± 2.8		23	56.8 ± 2.9	
DRB4-	39	58.1 ± 2.5	0.444	36	57.8 ± 2.6	0.612
DRB4+	75	60.3 ± 1.6		63	59.3 ± 1.7	
DRB5-	77	59.4 ± 1.6	0.842	69	59.0 ± 1.7	0.825
DRB5+	37	60.0 ± 2.5		30	58.3 ± 2.8	

[†] Primary tumors obtained from breast carcinoma patients with outcome information.
[‡] Primary tumors from outcome set that were analyzed by immunohistochemistry.
[§] *P*-value (two-tailed) calculated using one-way analysis of variance.

Table AI.2: HLA-DRB allele associations with estrogen receptor (ER) expression in breast carcinoma patients.

	Primary Tumor Outcome Set[†]			Primary Tumor IHC Set[‡]		
	N	**Mean ER (± SEM)**	***P*[§]**	**N**	**Mean ER (± SEM)**	***P*[§]**
Total	114	36.0 ± 5.4	-	99	38.0 ± 6.1	-
DRB1*01-	98	33.7 ± 5.9	0.141	85	36.5 ± 6.7	0.322
DRB1*01+	16	49.8 ± 13.0		14	47.0 ± 14.0	
DRB1*03-	96	38.7 ± 6.2	0.470	83	41.2 ± 7.0	0.320
DRB1*03+	18	21.4 ± 9.1		16	21.4 ± 10.3	
DRB1*04-	65	37.8 ± 7.9	0.559	59	38.2 ± 8.5	0.234
DRB1*04+	49	33.6 ± 7.1		40	37.7 ± 8.5	
DRB1*0401-	89	37.9 ± 6.5	0.646	79	38.9 ± 7.2	0.144
DRB1*0401+	25	29.1 ± 8.3		20	34.2 ± 10.0	
DRB1*07-	83	34.4 ± 5.8	0.782	72	36.3 ± 6.5	0.432
DRB1*07+	31	40.2 ± 12.7		27	42.5 ± 14.5	
DRB1*11-	96	35.7 ± 5.8	0.991	82	37.6 ± 6.6	0.908
DRB1*11+	18	37.8 ± 15.6		17	39.7 ± 16.4	
DRB1*13-	93	34.2 ± 5.4	0.907	78	36.4 ± 6.3	0.922
DRB1*13+	21	44.1 ± 17.3		21	44.1 ± 17.3	
DRB1*15-	82	39.3 ± 6.7	0.226	73	42.1 ± 7.5	0.180
DRB1*15+	32	27.5 ± 8.6		26	26.3 ± 9.9	
DRB3-	59	34.2 ± 6.4	0.916	50	34.8 ± 7.3	0.724
DRB3+	55	38.0 ± 8.9		49	41.2 ± 9.9	
DRB3*01-	89	34.9 ± 5.7	0.784	76	36.9 ± 6.4	0.960
DRB3*01+	25	40.0 ± 14.5		23	41.6 ± 15.7	
DRB3*02-	89	36.3 ± 6.1	0.913	76	38.3 ± 7.0	0.859
DRB3*02+	25	34.9 ± 11.9		23	37.0 ± 12.8	
DRB4-	39	29.8 ± 8.2	0.403	36	28.5 ± 8.5	0.305
DRB4+	75	39.2 ± 7.0		63	43.4 ± 8.2	
DRB5-	77	39.2 ± 7.0	0.387	69	41.8 ± 7.8	0.308
DRB5+	37	29.4 ± 8.0		30	29.2 ± 9.3	

[†] Primary tumors obtained from breast carcinoma patients with outcome information.
[‡] Primary tumors from outcome set that were analyzed by immunohistochemistry.
[§] *P*-value (two-tailed) calculated using Mann-Whitney U test.

Table AI.3: HLA-DRB allele associations with progesterone receptor (PR) expression in breast carcinoma patients.

	Primary Tumor Outcome Set[†]			Primary Tumor IHC Set[‡]		
	N	**Mean PR (± SEM)**	***P*[§]**	**N**	**Mean PR (± SEM)**	***P*[§]**
Total	114	61.1 ± 12.9	-	99	59.6 ± 14.3	-
DRB1*01-	98	57.8 ± 13.3	0.213	85	55.8 ± 14.6	0.393
DRB1*01+	16	81.5 ± 43.5		14	82.7 ± 49.8	
DRB1*03-	96	66.6 ± 15.1	0.807	83	66.3 ± 16.9	0.768
DRB1*03+	18	32.2 ± 10.6		16	24.6 ± 6.9	
DRB1*04-	65	66.0 ± 20.8	0.489	59	63.5 ± 22.5	0.299
DRB1*04+	49	54.7 ± 11.8		40	53.8 ± 12.8	
DRB1*0401-	89	63.2 ± 15.8	0.114	79	60.7 ± 17.3	0.089
DRB1*0401+	25	53.9 ± 16.6		20	55.3 ± 19.5	
DRB1*07-	83	57.2 ± 11.5	0.428	72	56.3 ± 12.6	0.243
DRB1*07+	31	71.7 ± 36.3		27	68.3 ± 41.0	
DRB1*11-	96	67.5 ± 15.1	0.289	82	66.0 ± 17.1	0.543
DRB1*11+	18	27.1 ± 10.1		17	28.6 ± 10.6	
DRB1*13-	93	61.7 ± 15.3	0.846	78	59.8 ± 17.6	0.748
DRB1*13+	21	58.7 ± 17.9		21	58.7 ± 17.9	
DRB1*15-	82	65.1 ± 17.0	0.932	73	64.8 ± 18.7	0.924
DRB1*15+	32	50.9 ± 14.7		26	44.9 ± 14.6	
DRB3-	59	78.7 ± 23.5	0.698	50	77.3 ± 26.9	1.000
DRB3+	55	42.3 ± 8.4		49	41.5 ± 8.9	
DRB3*01-	89	65.8 ± 16.0	0.451	76	65.4 ± 18.2	0.431
DRB3*01+	25	44.6 ± 13.8		23	40.4 ± 13.7	
DRB3*02-	89	65.8 ± 16.0	0.696	76	64.2 ± 18.1	0.709
DRB3*02+	25	44.5 ± 13.4		23	44.5 ± 14.4	
DRB4-	39	61.4 ± 20.5	0.837	36	54.5 ± 21.1	0.870
DRB4+	75	61.0 ± 16.5		63	62.5 ± 19.1	
DRB5-	77	66.9 ± 18.0	0.851	69	66.1 ± 19.7	0.799
DRB5+	37	49.2 ± 13.1		30	44.6 ± 13.2	

[†] Primary tumors obtained from breast carcinoma patients with outcome information.
[‡] Primary tumors from outcome set that were analyzed by immunohistochemistry.
[§] *P*-value (two-tailed) calculated using Mann-Whitney U test.

Table AI.4: HLA-DRB allele associations with primary tumor diameter in breast carcinoma patients.

	Primary Tumor Outcome Set[†]			Primary Tumor IHC Set[‡]		
	N	Mean PR (± SEM)	$P^{§}$	N	Mean PR (± SEM)	$P^{§}$
Total	111	3.3 ± 0.2	-	96	3.3 ± 0.2	-
DRB1*01-	97	3.4 ± 0.2	0.644	84	3.3 ± 0.3	0.833
DRB1*01+	14	2.9 ± 0.3		12	2.8 ± 0.3	
DRB1*03-	94	3.3 ± 0.2	0.539	81	3.3 ± 0.3	0.492
DRB1*03+	17	3.3 ± 0.4		15	3.3 ± 0.4	
DRB1*04-	64	3.2 ± 0.2	0.274	58	3.1 ± 0.2	0.394
DRB1*04+	47	3.4 ± 0.4		38	3.6 ± 0.5	
DRB1*0401-	87	3.4 ± 0.2	0.036	77	3.4 ± 0.2	0.060
DRB1*0401+	24	2.9 ± 0.05		19	2.9 ± 0.6	
DRB1*07-	80	3.4 ± 0.3	0.818	69	3.4 ± 0.3	0.610
DRB1*07+	31	3.1 ± 0.3		27	3.0 ± 0.3	
DRB1*11-	93	3.2 ± 0.2	0.648	79	3.2 ± 0.2	0.510
DRB1*11+	18	3.6 ± 0.7		17	3.6 ± 0.7	
DRB1*13-	91	3.3 ± 0.2	0.506	76	3.3 ± 0.3	0.349
DRB1*13+	20	3.3 ± 0.4		20	3.3 ± 0.4	
DRB1*15-	79	3.2 ± 0.2	0.722	70	3.2 ± 0.2	0.511
DRB1*15+	32	3.4 ± 0.5		26	3.4 ± 0.6	
DRB3-	58	3.1 ± 0.3	0.050	49	3.1 ± 0.3	0.076
DRB3+	53	3.5 ± 0.3		47	3.5 ± 0.3	
DRB3*01-	87	3.2 ± 0.2	0.128	74	3.2 ± 0.3	0.080
DRB3*01+	24	3.6 ± 0.4		22	3.6 ± 0.4	
DRB3*02-	86	3.2 ± 0.2	0.076	73	3.2 ± 0.3	0.113
DRB3*02+	25	3.7 ± 0.5		23	3.7 ± 0.5	
DRB4-	38	3.1 ± 0.3	0.578	35	3.1 ± 0.3	0.350
DRB4+	73	3.4 ± 0.3		61	3.4 ± 0.3	
DRB5-	74	3.3 ± 0.2	0.564	66	3.3 ± 0.3	0.587
DRB5+	37	3.3 ± 0.4		30	3.3 ± 0.5	

[†] Primary tumors obtained from breast carcinoma patients with outcome information.
[‡] Primary tumors from outcome set that were analyzed by immunohistochemistry.
[§] *P*-value (two-tailed) calculated using Mann-Whitney U test.

Table AI.5: HLA-DRB allele associations with lymph node metastasis in breast carcinoma patients.

	Primary Tumor Outcome Set[†]			Primary Tumor IHC Set[‡]		
	N	% LN+	*P*[§]	N	% LN+	*P*[§]
Total	113	50.4	-	98	54.1	-
DRB1*01-	97	52.6	0.264	84	57.1	0.136
DRB1*01+	16	37.5		14	35.7	
DRB1*03-	96	51.0	0.762	83	55.4	0.531
DRB1*03+	17	47.1		15	46.7	
DRB1*04-	64	48.4	0.626	58	50.0	0.329
DRB1*04+	49	53.1		40	60.0	
DRB1*0401-	88	50.0	0.860	78	53.8	0.926
DRB1*0401+	25	52.0		20	55.0	
DRB1*07-	82	50.0	0.878	71	53.5	0.857
DRB1*07+	31	51.6		27	55.6	
DRB1*11-	95	46.3	0.044	81	49.4	0.042
DRB1*11+	18	72.2		17	76.5	
DRB1*13-	93	47.3	0.151	78	51.3	0.272
DRB1*13+	20	65.0		20	65.0	
DRB1*15-	81	56.8	0.032	72	59.7	0.062
DRB1*15+	32	34.4		26	38.5	
DRB3-	59	39.0	0.011	50	44.0	0.041
DRB3+	54	63.0		48	64.6	
DRB3*01-	89	48.3	0.384	76	52.6	0.592
DRB3*01+	24	58.3		22	59.1	
DRB3*02-	88	43.2	0.004	75	46.7	0.008
DRB3*02+	25	76.0		23	78.3	
DRB4-	38	50.0	0.947	35	51.4	0.694
DRB4+	75	50.7		63	55.6	
DRB5-	76	57.9	0.023	68	60.3	0.063
DRB5+	37	35.1		30	40.0	

[†] Primary tumors obtained from breast carcinoma patients with outcome information.
[‡] Primary tumors from outcome set that were analyzed by immunohistochemistry.
[§] *P*-value (two-tailed) calculated using Pearson's Chi square or Fisher's exact test for 2x2 tables when at least one expected count was ≤ 5.

Table AI.6: HLA-DRB allele associations with histological tumor grade in breast carcinoma patients.

	Primary Tumor Outcome Set[†]			Primary Tumor IHC Set[‡]		
	N	% Histological Grade III	$P^{§}$	N	% Histological Grade III	$P^{§}$
Total	105	35.2	-	91	38.5	-
DRB1*01-	92	37.0	0.536	80	40.0	0.521
DRB1*01+	13	23.1		11	27.3	
DRB1*03-	87	33.3	0.369	75	36.0	0.296
DRB1*03+	18	44.4		16	50.0	
DRB1*04-	60	30.0	0.194	54	33.3	0.224
DRB1*04+	45	42.2		37	45.9	
DRB1*0401-	83	34.9	0.901	74	39.2	0.766
DRB1*0401+	22	36.4		17	35.3	
DRB1*07-	76	39.5	0.141	66	42.4	0.207
DRB1*07+	29	24.1		25	28.0	
DRB1*11-	88	35.2	0.996	75	38.7	0.931
DRB1*11+	17	35.3		16	37.5	
DRB1*13-	86	36.0	0.712	72	40.3	0.488
DRB1*13+	19	31.6		19	31.6	
DRB1*15-	77	36.4	0.689	68	39.7	0.675
DRB1*15+	28	32.1		23	34.8	
DRB3-	53	37.7	0.589	45	42.2	0.466
DRB3+	52	32.7		46	34.8	
DRB3*01-	80	35.0	0.927	68	38.2	0.939
DRB3*01+	25	36.0		23	39.1	
DRB3*02-	81	35.8	0.824	69	40.6	0.462
DRB3*02+	24	33.3		22	31.8	
DRB4-	35	40.0	0.470	33	42.4	0.558
DRB4+	70	32.9		58	36.2	
DRB5-	72	34.7	0.870	64	37.5	0.772
DRB5+	33	36.4		27	40.7	

[†] Primary tumors obtained from breast carcinoma patients with outcome information.
[‡] Primary tumors from outcome set that were analyzed by immunohistochemistry.
[§] *P*-value (two-tailed) calculated using Pearson's Chi square or Fisher's exact test for 2x2 tables when at least one expected count was ≤ 5.

Appendix II

Associations of clinicopathological parameters with patient survival in primary invasive breast carcinomas

AII Associations of clinicopathological parameters with patient survival in primary invasive breast carcinomas

The prognostic significance of clinicopathological parameters of primary invasive breast carcinomas utilized in the studies described in Chapters 3-5 was assessed. Associations with recurrence-free survival (Table AII.1), distant recurrence-free survival (Table AII.2) and disease-specific survival (Table AII.3) were determined by Kaplan Meier analysis with log rank statistic.

Table AII.1: Kaplan Meier Analysis of Clinicopathological parameters with Recurrence-free survival[a].

Prognostic Parameter	N	% Survival	Log Rank	p
Surgery	25	64.0	0.03	0.866
Surgery + Adjuvant	87	58.6		
IDC	86	52.9	4.75	**0.029**
ILC or Mixed	24	84.0		
Grade I	16	81.3	3.18	0.204
Grade II	52	57.7		
Grade III	37	54.1		
LN^-	56	75.0	6.24	**0.013**
LN^+	57	47.4		
Size ≤2cm	28	78.6	5.45	**0.020**
Size >2cm	53	53.0		
cTNM Stage 1	16	81.3	117.95	**<0.0001**
cTNM Stage 2	73	65.8		
cTNM Stage 3	18	27.8		
cTNM Stage 4	3	0.0		
Dx Age <50	36	50.0	1.53	0.466
Dx Age 50-59	18	61.1		
Dx Age ≥60	60	66.7		
ER<10fmol/mg	48	43.8	8.19	**0.004**
ER ≥10fmol/mg	66	72.7		
PR<10fmol/mg	39	53.9	2.89	0.089
PR ≥10fmol/mg	75	64.0		
Her-2/neu^- (0-2)	70	61.4	2.94	0.087
Her-2/neu^+ (3)	19	42.1		

[a] Recurrence-free survival = Time to Recurrence (Distant or Regional)

Table AII.2: Kaplan Meier Analysis of Clinicopathological parameters with Distant recurrence-free survival[a].

Prognostic Parameter	N	% Survival	Log Rank	p
Surgery	25	72.0	0.00	0.966
Surgery + Adjuvant	87	65.5		
IDC	86	60.5	5.74	**0.017**
ILC or Mixed	24	91.7		
Grade I	16	93.8	5.09	0.078
Grade II	52	67.3		
Grade III	37	56.8		
LN^-	56	80.4	5.54	**0.019**
LN^+	57	56.1		
Size ≤2cm	28	82.1	4.09	**0.043**
Size >2cm	53	61.5		
cTNM Stage 1	16	81.3	114.45	**<0.0001**
cTNM Stage 2	73	72.6		
cTNM Stage 3	18	44.4		
cTNM Stage 4	3	0.0		
Dx Age <50	36	52.8	3.91	0.142
Dx Age 50-59	18	66.7		
Dx Age ≥60	60	76.7		
ER<10fmol/mg	48	47.9	12.29	**0.0005**
ER ≥10fmol/mg	66	81.8		
PR<10fmol/mg	39	56.4	4.98	**0.026**
PR ≥10fmol/mg	75	73.3		
Her-2/neu^- (0-2)	70	68.6	2.20	0.138
Her-2/neu^+ (3)	19	52.6		

[a] Distant recurrence-free survival = Time to distant metastasis

Table AII.3: Kaplan Meier Analysis of Clinicopathological parameters with Disease-specific survival[a].

Prognostic Parameter	N	% Survival	Log Rank	p
Surgery	19	63.2	0.44	0.506
Surgery + Adjuvant	78	65.4		
IDC	75	62.7	0.82	0.364
ILC or Mixed	20	75.0		
Grade I	14	78.6	1.14	0.565
Grade II	43	65.1		
Grade III	34	61.8		
LN^-	48	75.0	1.76	0.185
LN^+	50	58.0		
Size ≤2cm	27	85.2	4.63	**0.032**
Size >2cm	70	58.6		
cTNM Stage 1	15	73.3	24.8	**<0.0001**
cTNM Stage 2	64	71.9		
cTNM Stage 3	14	50.0		
cTNM Stage 4	3	0.0		
Dx Age <50	33	60.6	0.57	0.751
Dx Age 50-59	18	61.1		
Dx Age ≥60	48	70.8		
ER<10fmol/mg	46	50.0	8.00	**0.005**
ER≥10fmol/mg	53	79.3		
PR<10fmol/mg	34	44.1	12.60	**0.0004**
PR≥10fmol/mg	65	76.9		
Her-2/neu^- (0-2)	60	66.7	0.70	0.403
Her-2/neu^+ (3)	17	58.8		

[a] Breast cancer-free survival = Time to death from breast cancer. Patients who died of other causes or were alive with other malignancy were omitted from analysis.

Appendix III

Determination of Hardy-Weinberg equilibrium and associations of cytokine SNPs with intratumoral cytokine mRNA levels

Supplementary Tables for Chapter 6

AIII Determination of Hardy-Weinberg equilibrium and associations of cytokine SNPs with intratumoral cytokine mRNA levels

To ensure cytokine SNPs followed Hardy Weinberg equilibrium observed and expected frequencies were compared by Chi-Square analysis (Table AIII.1). To determine the validity of assessing combined primary and recurrent tumors, associations of cytokine SNPs with relative intratumoral cytokine mRNA levels were compared in primary tumors and the combined primary and recurrent tumor subsets (Tables AIII.2-10).

Table AIII.1: Comparison of observed and expected SNP genotypes in breast carcinoma patients to determine if allele distribution follows Hardy Weinberg equilibrium.

SNP Genotype	Observed		Expected*		$\chi^{2\dagger}$	*P*-value
	N	%	N	%		
IL-1β -511 C/C	34	57.6	31.3	53.1	3.1	0.209
IL-1β -511 C/T	18	30.5	23.3	39.5		
IL-1β -511 T/T	7	11.9	4.3	7.4		
Total	59	100.0	59.0	100.0		
IL-1β +3962T/T	0	0.0	3.1	5.2	1.8	0.174
IL-1β +3962 T/C	27	45.8	20.8	35.3		
IL-1β +3962 C/C	32	54.2	35.1	59.5		
Total	59	100.0	59.0	100.0		
IL-1R pst1 1970 C/C	25	41.7	26.0	43.3	0.3	0.848
IL-1R pst1 1970 C/T	29	48.3	27.0	45.0		
IL-1R pst1 1970 T/T	6	10.0	7.0	11.7		
Total	60	100.0	60.0	100.0		
IL-1RN mspa1 11100C/C	6	10.0	6.3	10.6	0.0	0.982
IL-1RN mspa1 11100T/C	27	45.0	26.3	43.9		
IL-1RN mspa1 11100T/T	27	45.0	27.3	45.6		
Total	60	100.0	60.0	100.0		
IL-12 -1188 A/A	35	60.3	34.9	60.2	0.0	0.997
IL-12 -1188 C/A	20	34.5	20.2	34.8		
IL-12 -1188 C/C	3	5.2	2.9	5.0		
Total	58	100.0	58.0	100.0		
IFNγ +874 A/A	16	27.1	16.8	28.5	0.2	0.916
IFNγ +874 A/T	31	52.5	29.4	49.8		
IFNγ +874 T/T	12	20.3	12.8	21.7		
Total	59	100.0	59.0	100.0		
TGF-β1 Codon 10 C/C	5	8.9	7.5	13.4	2.1	0.355
TGF-β1 Codon 10 C/T	31	55.4	26.0	46.4		
TGF-β1 Codon 10 T/T	20	35.7	22.5	40.2		
Total	56	100.0	56.0	100.0		
TGF-β1 Codon 25 G/G	48	85.7	48.3	86.2	0.0	0.825
TGF-β1 Codon 25 G/C	8	14.3	7.4	13.3		
TGF-β1 Codon 25 C/C	0	0.0	0.3	0.5		
Total	56	100.0	56.0	100.0		
TNF-α -308 G/G	38	63.3	39.2	65.3	1.0	0.606
TNF-α-308 G/A	21	35.0	18.6	31.0		
TNF-α -308 A/A	1	1.7	2.2	3.7		
Total	60	100.0	60.0	100.0		
TNF-α -238 G/G	55	91.7	54.2	90.3	3.7	0.155
TNF-α -238 G/A	4	6.7	5.7	9.5		
TNF-α -238 A/A	1	1.7	0.2	0.3		
Total	60	100.0	60.0	100.0		
IL-2 -330 T/T	28	46.7	29.4	49.0	0.7	0.690
IL-2 -330 T/G	28	46.7	25.2	42.0		
IL-2 -330 G/G	4	6.7	5.4	9.0		
Total	60	100.0	60.0	100.0		
IL-2 +166 G/G	28	46.7	28.7	47.8	0.2	0.914
IL-2 +166 G/T	27	45.0	25.6	42.7		

SNP Genotype	Observed		Expected*		$\chi^{2\dagger}$	*P*-value
	N	%	N	%		
IL-2 +166 T/T	5	8.3	5.7	9.5		
Total	60	100.0	60.0	100.0		
IL-4 -1098 T/T	52	86.7	50.4	84.0	7.6	0.023
IL-4 -1098 T/G	6	10.0	9.2	15.3		
IL-4 -1098 G/G	2	3.3	0.4	0.7		
Total	60	100.0	60.0	100.0		
IL-4 -590 T/T	1	1.7	1.5	2.5	0.2	0.889
IL-4 -590 T/C	17	28.3	16.0	26.7		
IL-4 -590 C/C	42	70.0	42.5	70.8		
Total	60	100.0	60.0	100.0		
IL-4 -33 T/T	1	1.7	1.5	2.6	0.2	0.883
IL-4 -33 T/C	17	28.8	15.9	27.0		
IL-4 -33 C/C	41	69.5	41.5	70.4		
Total	59	100.0	59.0	100.0		
IL-6 -174 G/G	22	36.7	24.7	41.2	2.3	0.317
IL-6 -174 G/C	33	55.0	27.6	46.0		
IL-6 -174 C/C	5	8.3	7.7	12.8		
Total	60	100.0	60.0	100.0		
IL-6 nt565 G/G	22	36.7	25.4	42.3	3.7	0.160
IL-6 nt565 G/A	34	56.7	27.3	45.5		
IL-6 nt565 A/A	4	6.7	7.4	12.3		
Total	60	100.0	60.0	100.0		
IL-10 -1082 G/G	10	16.9	12.4	20.9	1.6	0.460
IL-10 -1082 G/A	34	57.6	29.3	49.6		
IL-10 -1082 A/A	15	25.4	17.4	29.4		
Total	59	100.0	59.0	100.0		
IL-10 -819 C/C	31	52.5	31.3	53.1	0.0	0.978
IL-10 -819 C/T	24	40.7	23.3	39.5		
IL-10 -819 T/T	4	6.8	4.3	7.4		
Total	59	100.0	59.0	100.0		
IL-10 -592 C/C	33	55.9	32.1	54.4	0.4	0.827
IL-10 -592 C/A	21	35.6	22.9	38.7		
IL-10 -592 A/A	5	8.5	4.1	6.9		
Total	59	100.0	59.0	100.0		

* Expected frequencies determined using Hardy-Weinberg equation ($1=p^2+2pq+q^2$, where p=proportion of common allele and q=proportion of rare allele and p^2 represents common allele homozygotes, pq represents heterozygotes and q^2 represents rare allele homozygotes).

†The Chi-square statistic was computed by squaring the residual for each genotype, dividing by its expected value, and summing for all genotypes.

Table AIII.2: Associations of IL-2 SNPs with intratumoral IL-2 mRNA* in breast carcinoma tumors

	Primary Tumors				Primary and Secondary Tumors			
	N	**IL-2**	**SEM**	**P**	**N**	**IL-2**	**SEM**	**P**
IL-2 -330 T-	4	3.8	1.5	0.149	4	3.8	1.5	0.156
IL-2 -330 T+	48	13.4	1.8		56	12.8	1.6	
IL-2 -330 G-	24	12.7	2.0	0.451	28	12.8	2.0	0.299
IL-2 -330 G+	28	12.5	2.7		32	11.7	2.4	
IL-2 -330 T/T	24	12.7	2.0	0.313	28	12.8	2.0	0.278
IL-2 -330 T/G	24	14.0	3.0		28	12.8	2.6	
IL-2 -330 G/G	4	3.8	1.5		4	3.8	1.5	
IL-2 +166 G-	5	6.2	3.0	0.210	5	6.2	3.0	0.259
IL-2 +166 G+	47	13.3	1.8		55	12.7	1.7	
IL-2 +166 T-	23	14.1	2.4	0.265	28	14.0	2.2	0.106
IL-2 +166 T+	29	11.4	2.3		32	10.5	2.2	
IL-2 +166 G/G	23	14.1	2.4	0.323	28	14.0	2.2	0.206
IL-2 +166 G/T	24	12.5	2.7		27	11.4	2.5	
IL-2 +166 T/T	5	6.2	3.0		5	6.2	3.0	
IL-2 -330+166 TG-	23	9.3	2.6	**0.017**	25	8.6	2.5	**0.006**
IL-2 -330+166 TG+	29	15.3	2.1		35	14.7	1.9	
IL-2 -330+166 GG-	24	12.7	2.0	0.451	28	12.8	2.0	0.299
IL-2 -330+166 GG+	28	12.5	2.7		32	11.7	2.4	
IL-2 -330+166 TT-	23	14.1	2.4	0.265	28	14.0	2.2	0.106
IL-2 -330+166 TT+	29	11.4	2.3		32	10.5	2.2	
IL-2 -330+166 GG/GG	4	3.8	1.5	0.313	4	3.8	1.5	0.278
IL-2 -330+166 GG/x	24	14.0	3.0		28	12.8	2.6	
IL-2 -330+166 x/x	24	12.7	2.0		28	12.8	2.0	
IL-2 -330+166 TG/TG	9	15.7	3.0	0.052	12	15.8	3.1	**0.020**
IL-2 -330+166 TG/x	20	15.1	2.8		23	14.2	2.4	
IL-2 -330+166 x/x	23	9.3	2.6		25	8.6	2.5	
IL-2 -330+166 TT/TT	5	6.2	3.0	0.323	5	6.2	3.0	0.206
IL-2 -330+166 TT/x	24	12.5	2.7		27	11.4	2.5	
IL-2 -330+166 x/x	23	14.1	2.4		28	14.0	2.2	

* Relative mRNA levels determined using RT-PCR to amplify total RNA (1 μg) prepared from fresh-frozen breast carcinoma tissue. Relative amounts of cytokine mRNA were determined by normalizing spot densities of amplified products to β-actin spot density for each sample.

Table AIII.3: Associations of IFN-γ SNPs with intratumoral IFN-γ mRNA* in breast carcinoma tumors

	Primary Tumors				Primary and Secondary Tumors			
	N	**IFN-γ**	**SEM**	**P**	**N**	**IFN-γ**	**SEM**	**P**
IFN-γ +874 A/A	15	4.8	1.6	**0.028**	16	4.6	1.5	**0.010**
IFN-γ +874 A/T	25	17.2	3.7		31	16.9	3.1	
IFN-γ +874 T/T	11	6.6	1.4		12	6.4	1.3	
IFN-γ +874 A-	11	6.6	1.4	0.792	12	6.4	1.3	0.658
IFN-γ +874 A+	40	12.5	2.5		47	12.7	2.3	
IFN-γ +874 T-	15	4.8	1.6	**0.016**	16	4.6	1.5	**0.007**
IFN-γ +874 T+	36	13.9	2.7		43	13.9	2.4	

* Relative mRNA levels determined using RT-PCR to amplify total RNA (1 μg) prepared from fresh-frozen breast carcinoma tissue. Relative amounts of cytokine mRNA were determined by normalizing spot densities of amplified products to β-actin spot density for each sample.

Table AIII.4: Associations of IL-12 SNPs with intratumoral IL-12 mRNA* in breast carcinoma tumors

	Primary Tumors				Primary and Secondary Tumors			
	N	**IL-12**	**SEM**	**P**	**N**	**IL-12**	**SEM**	**P**
IL-12 -1188 A/A	29	8.3	1.6	0.771	34	7.4	1.4	0.959
IL-12 -1188 C/A	15	6.1	1.4		18	5.6	1.2	
IL-12 -1188 C/C	2	5.8	3.7		2	5.8	3.7	
IL-12 -1188 C-	29	8.3	1.6	0.473	34	7.4	1.4	0.774
IL-12 -1188 C+	17	6.0	1.3		20	5.6	1.1	
IL-12 -1188 A-	2	5.8	3.7	0.812	2	5.8	3.7	0.981
IL-12 -1188 A+	44	7.5	1.1		52	6.8	1.0	

* Relative mRNA levels determined using RT-PCR to amplify total RNA (1 μg) prepared from fresh-frozen breast carcinoma tissue. Relative amounts of cytokine mRNA were determined by normalizing spot densities of amplified products to β-actin spot density for each sample.

Table AIII.5: Associations of IL-4 and IL-4Ra SNPs with IL-4 mRNA* in breast carcinoma tumors

	Primary Tumors				Primary and Secondary Tumors			
	N	IL-4	SEM	P	N	IL-4	SEM	P
IL-4 -1098 T-	1	3.1	.	nc	2	1.6	1.6	nc
IL-4 -1098 T+	47	1.1	0.4		54	1.0	0.3	
IL-4 -1098 G-	43	1.0	0.4	nc	49	0.9	0.4	nc
IL-4 -1098 G+	5	2.6	1.1		7	1.8	0.9	
IL-4 -1098 T/T	43	1.0	0.4	nc	49	0.9	0.4	nc
IL-4 -1098 T/G	4	2.4	1.4		5	1.9	1.2	
IL-4 -1098 G/G	1	3.1	.		2	1.6	1.6	
IL-4 -590 T-	32	1.4	0.6	0.830	38	1.1	0.5	0.808
IL-4 -590 T+	16	0.7	0.2		18	0.8	0.2	
IL-4 -590 C-	1	2.0	.	0.333	1	2.0	.	0.321
IL-4 -590 C+	47	1.1	0.4		55	1.0	0.3	
IL-4 -590 T/T	1	2.0	.	0.381	1	2.0	.	0.490
IL-4 -590 T/C	15	0.6	0.2		17	0.7	0.2	
IL-4 -590 C/C	32	1.4	0.6		38	1.1	0.5	
IL-4 -33 T-	31	1.3	0.6	0.635	37	1.1	0.5	0.368
IL-4 -33 T+	16	0.9	0.3		18	0.9	0.3	
IL-4 -33 C-	1	2.0	.	0.333	1	2.0	.	0.321
IL-4 -33 C+	47	1.1	0.4		55	1.0	0.3	
IL-4 -33 T/T	1	2.0	.	0.431	1	2.0	.	0.337
IL-4 -33 T/C	15	0.8	0.3		17	0.9	0.3	
IL-4 -33 C/C	31	1.3	0.6		37	1.1	0.5	
IL-4 -1098-590-33 TTT-	32	1.4	0.6	nc	38	1.1	0.5	nc
IL-4 -1098-590-33 TTT+	15	0.8	0.2		17	0.8	0.2	
IL-4 -1098-590-33 TCC-	2	2.6	0.6	nc	3	1.7	0.9	nc
IL-4 -1098-590-33 TCC+	46	1.1	0.4		53	1.0	0.4	
IL-4 -1098-590-33 GCT-	47	1.1	0.4	nc	55	1.0	0.3	nc
IL-4 -1098-590-33 GCT+	1	3.1	.		1	3.1	.	
IL-4 -1098-590-33 GCC-	43	1.0	0.4	nc	49	0.9	0.4	nc
IL-4 -1098-590-33 GCC+	5	2.6	1.1		7	1.8	0.9	
IL-4 -1098-590-33 GCC/GCC	0	-	-	nc	1	0.0	.	nc
IL-4 -1098-590-33 GCC/x	5	2.6	1.1		6	2.1	1.0	
IL-4 -1098-590-33 x/x	42	1.0	0.4		48	0.9	0.4	
IL-4 -1098-590-33 TCC/TCC	27	1.1	0.6	nc	31	1.0	0.6	nc
IL-4 -1098-590-33 TCC/x	18	1.1	0.4		21	1.0	0.3	
IL-4 -1098-590-33 x/x	2	2.6	0.6		3	1.7	0.9	
IL-4 -1098-590-33 TTT/TTT	1	2.0	.	nc	1	2.0	.	nc
IL-4 -1098-590-33 TTT/x	14	0.7	0.2		16	0.7	0.2	
IL-4 -1098-590-33 x/x	32	1.4	0.6		38	1.1	0.5	

*Relative mRNA levels determined using RT-PCR to amplify total RNA (1 μg) prepared from fresh-frozen breast carcinoma tissue. Relative amounts of cytokine mRNA were determined by normalizing spot densities of amplified products to β-actin spot density for each sample.

nc=Not Calculated; IL-4 -1098 genotype did not follow Hardy-Weinberg equilibrium (Table A3.1).

Table AIII.6: Associations of IL-10 SNPs with intratumoral IL-10 mRNA* in breast carcinoma tumors

	Primary Tumors				Primary and Secondary Tumors			
	N	IL-10	SEM	P	N	IL-10	SEM	P
IL-10 -1082 G-	15	20.7	3.6	0.479	15	20.7	3.6	0.609
IL-10 -1082 G+	32	23.4	2.1		39	22.4	1.9	
IL-10 -1082 A-	7	25.3	4.8	0.510	8	23.9	4.4	0.648
IL-10 -1082 A+	41	21.7	1.9		47	21.3	1.8	
IL-10 -1082 G/G	7	25.3	4.8	0.706	8	23.9	4.4	0.841
IL-10 -1082 G/A	25	22.8	2.3		31	22.0	2.1	
IL-10 -1082 A/A	15	20.7	3.6		15	20.7	3.6	
IL-10 -819 C-	4	22.6	6.6	0.985	4	22.6	6.6	0.861
IL-10 -819 C+	43	22.5	1.9		50	21.8	1.7	
IL-10 -819 T-	23	23.6	2.4	0.599	27	22.1	2.2	0.946
IL-10 -819 T+	25	21.0	2.6		28	21.3	2.5	
IL-10 -819 C/C	23	23.6	2.4	0.926	27	22.1	2.2	0.980
IL-10 -819 C/T	20	21.4	3.0		23	21.6	2.8	
IL-10 -819 T/T	4	22.6	6.6		4	22.6	6.6	
IL-10 -592 C-	5	20.1	5.7	0.700	5	20.1	5.7	0.817
IL-10 -592 C+	42	22.8	1.9		49	22.1	1.8	
IL-10 -592 A-	25	22.0	2.4	0.579	29	20.8	2.2	0.353
IL-10 -592 A+	22	23.2	2.7		25	23.2	2.6	
IL-10 -592 C/C	25	22.0	2.4	0.688	29	20.8	2.2	0.540
IL-10 -592 C/A	17	24.1	3.2		20	24.0	3.0	
IL-10 -592 A/A	5	20.1	5.7		5	20.1	5.7	
IL-10 -1082-819-592 ACA-	46	22.8	1.8	0.468	53	22.1	1.7	0.481
IL-10 -1082-819-592 ACA+	1	10.1	.		1	10.1	.	
IL-10 -1082-819-592 ACC-	22	21.9	2.6	0.865	26	21.7	2.4	0.945
IL-10 -1082-819-592 ACC+	25	23.1	2.5		28	22.0	2.4	
IL-10 -1082-819-592 ATA-	26	22.3	2.3	0.748	30	21.1	2.1	0.486
IL-10 -1082-819-592 ATA+	21	22.8	2.9		24	22.9	2.7	
IL-10 -1082-819-592 ATC-	45	23.3	1.8	**0.046**	52	22.5	1.7	0.050
IL-10 -1082-819-592 ATC+	2	5.9	2.2		2	5.9	2.2	
IL-10 -1082-819-592 GCA-	46	22.4	1.8	0.553	53	21.7	1.7	0.519
IL-10 -1082-819-592 GCA+	1	30.4	.		1	30.4	.	
IL-10 -1082-819-592 GCC-	17	21.6	3.2	0.740	17	21.6	3.2	0.918
IL-10 -1082-819-592 GCC+	30	23.1	2.2		37	22.0	2.0	
IL-10 -1082-819-592 GTC-	46	22.5	1.8	0.809	53	21.8	1.7	0.741
IL-10 -1082-819-592 GTC+	1	26.2	.		1	26.2	.	
IL-10 -1082-819-592 GCC/GCC	7	25.3	4.8	0.813	8	23.9	4.4	0.918
IL-10 -1082-819-592 GCC/x	23	22.4	2.5		29	21.5	2.2	
IL-10 -1082-819-592 x/x	17	21.6	3.2		17	21.6	3.2	
IL-10 -1082-819-592 ACC/ACC	2	11.8	3.9	0.353	2	11.8	3.9	0.407
IL-10 -1082-819-592 ACC/x	23	24.1	2.6		26	22.8	2.5	
IL-10 -1082-819-592 x/x	22	21.9	2.6		26	21.7	2.4	
IL-10 -1082-819-592 ATA/ATA	4	22.6	6.6	0.947	4	22.6	6.6	0.785
IL-10 -1082-819-592 ATA/x	17	22.9	3.3		20	23.0	3.0	
IL-10 -1082-819-592 x/x	26	22.3	2.3		30	21.1	2.1	

* Relative mRNA levels determined using RT-PCR to amplify total RNA (1 μg) prepared from fresh-frozen breast carcinoma tissue. Relative amounts of cytokine mRNA were determined by normalizing spot densities of amplified products to β-actin spot density for each sample.

Table AIII.7: Associations of TGF-β SNPs with intratumoral TGF-β1 mRNA* in breast carcinoma tumors

	Primary Tumors				Primary and Secondary Tumors			
	N	TGF-β	SEM	P	N	TGF-β	SEM	P
TGF-β1 Codon 10 C-	16	52.4	8.0	0.586	19	54.4	7.3	0.697
TGF-β1 Codon 10 C+	29	55.9	3.9		33	54.5	3.6	
TGF-β1 Codon 10 T-	5	76.1	16.0	0.067	5	76.1	16.0	0.052
TGF-β1 Codon 10 T+	40	52.0	3.6		47	52.2	3.4	
TGF-β1 Codon 10 C/C	5	76.1	16.0	0.183	5	76.1	16.0	0.152
TGF-β1 Codon 10 C/T	24	51.7	3.0		28	50.7	2.8	
TGF-β1 Codon 10 T/T	16	52.4	8.0		19	54.4	7.3	
TGF-β1 Codon 25 G-	0			NC	52	54.5	3.5	NC
TGF-β1 Codon 25 G+	45	54.7	3.8					
TGF-β1 Codon 25 C-	38	53.0	3.8	0.702	44	53.5	3.5	0.931
TGF-β1 Codon 25 C+	7	63.8	13.5		8	60.0	12.2	
TGF-β1 Codon 25 G/G	38	53.0	3.8	0.684†	44	53.5	3.5	0.919†
TGF-β1 Codon 25 G/C	7	63.8	13.5	0.702‡	8	60.0	12.2	0.931‡
TGF-β1 Codon10Codon25 CG-	21	51.4	6.2	0.317	25	52.3	5.7	0.301
TGF-β1 Codon10Codon25 CG+	24	57.5	4.6		27	56.4	4.2	
TGF-β1 Codon10Codon25 CC-	38	53.0	3.8	0.702	44	53.5	3.5	0.931
TGF-β1 Codon10Codon25 CC+	7	63.8	13.5		8	60.0	12.2	
TGF-β1 Codon10Codon25 TG-	5	76.1	16.0	0.067	5	76.1	16.0	0.052
TGF-β1 Codon10Codon25 TG+	40	52.0	3.6		47	52.2	3.4	
TGF-β1 Codon10Codon25 TG/TG	16	52.4	8.0	0.183	19	54.4	7.3	0.152
TGF-β1 Codon10Codon25 TG/x	24	51.7	3.0		28	50.7	2.8	
TGF-β1 Codon10Codon25 x/x	5	76.1	16.0		5	76.1	16.0	
TGF-β1 Codon10Codon25 CG/CG	3	58.5	3.9	0.524	3	58.5	3.9	0.472
TGF-β1 Codon10Codon25 CG/x	21	57.4	5.2		24	56.2	4.7	
TGF-β1 Codon10Codon25 x/x	21	51.4	6.2		25	52.3	5.7	
TGF-β1 Codon10Codon25 CC/x	7	63.8	13.5	0.684†	8	60.0	12.2	0.919†
TGF-β1 Codon10Codon25 x/x	38	53.0	3.8	0.702‡	44	53.5	3.5	0.931‡

* Relative mRNA levels determined using RT-PCR to amplify total RNA (1 μg) prepared from fresh-frozen breast carcinoma tissue. Relative amounts of cytokine mRNA were determined by normalizing spot densities of amplified products to β-actin spot density for each sample.

†*P*-value calculated using Kruskal-Wallis H test.

‡*P*-value calculated using Mann-Whitney U test for 2 independent samples.

Table AIII.8: Associations of TNF-α SNPs with intratumoral TNF-α mRNA* in breast carcinoma tumors

	Primary Tumors				Primary and Secondary Tumors			
	N	TNF-α	SEM	P	N	TNF-α	SEM	P
TNF-α -308 G-	1	3.1	.	0.417	1	3.1	.	0.357
TNF-α -308 G+	47	1.8	0.3		55	1.6	0.3	
TNF-α -308 A-	33	1.4	0.3	0.143	35	1.4	0.2	0.618
TNF-α -308 A+	15	2.7	0.7		21	2.1	0.6	
TNF-α -308 G/G	33	1.4	0.3	0.263	35	1.4	0.2	0.502
TNF-α -308 G/A	14	2.7	0.8		20	2.1	0.6	
TNF-α -308 A/A	1	3.1	.		1	3.1	.	
TNF-α -238 G-	1	0.9	.	0.833	1	0.9	.	0.893
TNF-α -238 G+	47	1.8	0.3		55	1.7	0.3	
TNF-α -238 A-	44	1.8	0.3	0.957	51	1.7	0.3	0.738
TNF-α -238 A+	4	1.7	0.9		5	1.3	0.8	
TNF-α -238 G/G	44	1.8	0.3	0.934	51	1.7	0.3	0.935
TNF-α -238 G/A	3	1.9	1.2		4	1.5	1.0	
TNF-α -238 A/A	1	0.9	.		1	0.9	.	
TNF-α -308-238 GG-	2	2.0	1.1	0.64	3	1.3	0.9	0.891
TNF-α -308-238 GG+	46	1.8	0.3		53	1.7	0.3	
TNF-α -308-238 AG-	33	1.4	0.3	0.143	35	1.4	0.2	0.618
TNF-α -308-238 AG+	15	2.7	0.7		21	2.1	0.6	
TNF-α -308-238 GA-	44	1.8	0.3	0.957	51	1.7	0.3	0.738
TNF-α -308-238 GA+	4	1.7	0.9		5	1.3	0.8	
TNF-α -308-238 AG/AG	1	3.1	.	0.263	1	3.1	.	0.502
TNF-α -308-238 AG/x	14	2.7	0.8		20	2.1	0.6	
TNF-α -308-238 x/x	33	1.4	0.3		35	1.4	0.2	
TNF-α -308-238 GG/GG	29	1.4	0.3	0.36	31	1.4	0.3	0.797
TNF-α -308-238 GG/x	17	2.6	0.7		22	2.2	0.5	
TNF-α -308-238 x/x	2	2.0	1.1		3	1.3	0.9	
TNF-α -308-238 GA/GA	1	0.9	.	0.934	1	0.9	.	0.935
TNF-α -308-238 GA/x	3	1.9	1.2		4	1.5	1.0	
TNF-α -308-238 x/x	44	1.8	0.3		51	1.7	0.3	

* Relative mRNA levels determined using RT-PCR to amplify total RNA (1 μg) prepared from fresh-frozen breast carcinoma tissue. Relative amounts of cytokine mRNA were determined by normalizing spot densities of amplified products to β-actin spot density for each sample.

Table AIII.9: Associations of IL-1β,IL-1R and IL-1RN SNPs with IL-1β mRNA* in breast carcinomas

	Primary Tumors				Primary and Secondary Tumors			
	N	IL-1β	SEM	P	N	IL-1β	SEM	P
IL-1β -511 C-	7	17.9	3.1	0.529	7	17.9	3.1	0.61
IL-1β -511 C+	41	21.5	2.0		49	20.6	1.8	
IL-1β -511 T-	27	22.6	2.7	0.432	32	21.3	2.4	0.765
IL-1β -511 T+	20	18.4	2.1		23	18.6	1.8	
IL-1β -511 C/C	27	22.6	2.7	0.712	32	21.3	2.4	0.887
IL-1β -511 C/T	13	18.7	2.9		16	18.9	2.3	
IL-1β -511 T/T	7	17.9	3.1		7	17.9	3.1	
IL-1β +3962 T-	22	22.8	3.4	0.609	26	21.6	3.0	0.661
IL-1β +3962 T+	25	19.0	1.7		29	18.9	1.5	
IL-1β +3962 C-	1	38.3	.	0.213	2	26.1	12.3	0.743
IL-1β +3962 C+	46	20.4	1.8		53	19.9	1.6	
IL-1β +3962 T/C	24	18.2	1.5	0.360†	27	18.4	1.4	0.567†
IL-1β +3962 C/C	23	23.5	3.3	0.360‡	28	21.9	2.9	0.567‡
IL-1β -511+3962 CT-	23	22.6	3.2	0.685	27	21.5	2.9	0.755
IL-1β -511+3962 CT+	17	19.7	2.3		20	19.6	2.0	
IL-1β -511+3962 CC-	8	20.5	3.7	0.908	9	19.7	3.4	0.74
IL-1β -511+3962 CC+	32	21.6	2.5		38	20.9	2.2	
IL-1β -511+3962 TT-	39	21.5	2.1	1	46	20.7	1.9	0.979
IL-1β -511+3962 TT+	1	18.2	.		1	18.2	.	
IL-1β -511+3962 TC-	27	22.6	2.7	0.549	32	21.3	2.4	0.991
IL-1β -511+3962 TC+	13	18.9	3.0		15	19.3	2.6	
IL-1β -511+3962 CC/CC	10	27.5	6.2	0.521	12	24.2	5.7	0.905
IL-1β -511+3962 CC/x	22	19.0	2.1		26	19.3	1.8	
IL-1β -511+3962 x/x	8	20.5	3.7		9	19.7	3.4	
IL-1β -511+3962 TC/TC	6	17.9	3.7	0.746	6	17.9	3.7	0.759
IL-1β -511+3962 TC/x	7	19.8	4.9		9	20.2	3.8	
IL-1β -511+3962 x/x	27	22.6	2.7		32	21.3	2.4	
IL-1β -511+3962 CT/CT	1	38.3	.	0.324	2	26.1	12.3	0.877
IL-1β -511+3962 CT/x	16	18.6	2.1		18	18.8	1.9	
IL-1β -511+3962 x/x	23	22.6	3.2		27	21.5	2.9	
IL-1R pst1 1970 C/C	21	20.1	1.8	0.328	24	20.2	1.6	0.177
IL-1R pst1 1970 C/T	21	20.2	3.4		26	19.0	2.8	
IL-1R pst1 1970 T/T	6	26.6	5.4		6	26.6	5.4	
IL-1R pst1 1970 C-	6	26.6	5.4	0.213	6	26.6	5.4	0.177
IL-1R pst1 1970 C+	42	20.2	1.9		50	19.5	1.6	
IL-1R pst1 1970 T-	21	20.1	1.8	0.763	24	20.2	1.6	0.436
IL-1R pst1 1970 T+	27	21.6	2.9		32	20.4	2.5	
IL-1RN mspa1 11100C/C	5	24.4	6.3	0.304	6	22.6	5.5	0.397
IL-1RN mspa1 11100T/C	21	18.3	2.2		24	17.7	2.1	
IL-1RN mspa1 11100T/T	22	22.8	3.0		26	22.2	2.5	
IL-1RN mspa1 11100T-	5	24.4	6.3	0.338	6	22.6	5.5	0.56
IL-1RN mspa1 11100T+	43	20.6	1.9		50	20.0	1.7	
IL-1RN mspa1 11100C-	22	22.8	3.0	0.42	26	22.2	2.5	0.341
IL-1RN mspa1 11100C+	26	19.4	2.1		30	18.7	2.0	

* Relative mRNA levels determined using RT-PCR to amplify total RNA (1 μg) prepared from fresh-frozen breast carcinoma tissue. Relative amounts of cytokine mRNA were determined by normalizing spot densities of amplified products to β-actin spot density for each sample. †*P*-value calculated using Kruskal-Wallis H test. ‡*P*-value calculated using Mann-Whitney U test for 2 independent samples.

Table AIII.10: Associations of IL-6 SNPs with intratumoral IL-6 mRNA* in breast carcinoma tumors

	Primary Tumors				Primary and Secondary Tumors			
	N	**IL-6**	**SEM**	**P**	**N**	**IL-6**	**SEM**	**P**
IL-6 -174 G-	4	37.6	6.8	0.375	5	36.5	5.4	0.259
IL-6 -174 G+	44	29.1	3.1		51	28.0	2.8	
IL-6 -174 C-	17	32.1	5.9	0.73	19	32.3	5.3	0.467
IL-6 -174 C+	31	28.6	3.2		37	26.9	2.9	
IL-6 -174 G/G	17	32.1	5.9	0.551	19	32.3	5.3	0.304
IL-6 -174 G/C	27	27.3	3.5		32	25.4	3.2	
IL-6 -174 C/C	4	37.6	6.8		5	36.5	5.4	
IL-6 nt565 G-	3	42.7	6.3	0.229	4	40.1	5.2	0.163
IL-6 nt565 G+	45	29.0	3.0		52	27.9	2.7	
IL-6 nt565 A-	17	32.1	5.9	0.730	19	32.3	5.3	0.467
IL-6 nt565 A+	31	28.6	3.2		37	26.9	2.9	
IL-6 nt565 G/G	17	32.1	5.9	0.380	19	32.3	5.3	0.211
IL-6 nt565 G/A	28	27.1	3.4		33	25.3	3.1	
IL-6 nt565 A/A	3	42.7	6.3		4	40.1	5.2	
IL-6 -174nt565 GG-	4	37.6	6.8	0.375	5	36.5	5.4	0.259
IL-6 -174nt565 GG+	44	29.1	3.1		51	28.0	2.8	
IL-6 -174nt565 CG-	47	30.0	3.0	0.833	55	28.9	2.6	0.857
IL-6 -174nt565 CG+	1	22.0	.		1	22.0	.	
IL-6 -174nt565 CA-	17	32.1	5.9	0.730	19	32.3	5.3	0.467
IL-6 -174nt565 CA+	31	28.6	3.2		37	26.9	2.9	
IL-6 -174nt565 CA/CA	3	42.7	6.3	0.380	4	40.1	5.2	0.211
IL-6 -174nt565 CA/x	28	27.1	3.4		33	25.3	3.1	
IL-6 -174nt565 x/x	17	32.1	5.9		19	32.3	5.3	
IL-6 -174nt565 GG/GG	17	32.1	5.9	0.551	19	32.3	5.3	0.304
IL-6 -174nt565 GG/x	27	27.3	3.5		32	25.4	3.2	
IL-6 -174nt565 x/x	4	37.6	6.8		5	36.5	5.4	

* Relative mRNA levels determined using RT-PCR to amplify total RNA (1 μg) prepared from fresh-frozen breast carcinoma tissue. Relative amounts of cytokine mRNA were determined by normalizing spot densities of amplified products to β-actin spot density for each sample.